Transcript

Fourth Meeting of the Secretary's Advisory Committee on Xenotransplantation, U.S. Department of Health and Human Services

Monday, March 11, 2002

Holiday Inn Select
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P R O C E E D I N G S 8:00 A.M.

Agenda Item: Opening Remarks

DR. VANDERPOOL: I welcome all of you to this, the fourth meeting of the U.S. Department of Human Services Secretary's Advisory Committee for Xenotransplantation, which we call SACX. Now, a special welcome to all SACX members, to each and all, and also to each and all of our nonvoting members from the Centers for Disease Control, the Food and Drug Administration, the Health Resources and Services Administration, the National Institutes of Health and the Office of the Secretary of the DHHS. I also give special welcome to the outstanding panelist speakers who will be making presentations today and a special thanks to our Executive Director, Dr. Mary Groesch, as well as Dr. Megan Sykes and Dr. Dan Salomon, for bringing together such an outstanding list of speakers. Finally, I welcome the representatives of the public who are with us today, including members of the news media and special guests and acquaintances. Please note that there is a time for public comment set aside tomorrow at 9:15, and we urge interested parties to make brief statements and ask questions at that time.

This our fourth meeting is on the anniversary so to speak of our first, and I'm very pleased to be at a one-year anniversary period because I think we are hitting stride, and it's time to maintain stride to expeditiously do more work and take on a larger volume with probably less effort rather than more, and also to reach decision levels that we're called upon to reach. It's just quite incredible to read through the news items that Mary prepared for us in this meeting's agenda and see how many things regarding cloning, knockout pigs, and other rapidly advancing things are occurring.

In a few minutes our special science symposium will begin, a symposium that's been put together for the general purposes of our having a truly up-to-date understanding of crucial scientific developments, but more specifically for the purpose of one of our working groups. Our working groups, for the sake of those from the public, are groups that are formulating statements and papers for the scientific community and for the public. One is a statement of the state of science, and one involves issues involving informed consent. So today's program is especially designed to bring the state of the science working group into an acquaintance, even a further acquaintance, with critical and recent developments that will enable it to pool together its remarks and beliefs in its working paper.

Now, each of the subtopics and topics of this symposium are incredibly exciting, the physiology immune response, tolerance by artificial devices, transgenic pigs, including the announcements of the knockout pigs, cellular transplants and so on. I urge all our members to be preparing questions as the presentations are made. After each presentation there will be a very brief question and answer period to the presenter, followed by questions and responses and discussion by the committee and nonvoting members, but I urge all of us to formulate questions. For all of us nonscientific members, I urge us to develop questions, and I will give you a Vanderpool principle that no question is an embarrassing question because I think a lot of questions of nonscientists will reflect the kinds of issues the public are concerned about but don't formulate and don't have an opportunity to ask.

This day is slated to end no earlier than 7:00 P.M. I think there's a certain audacity to the schedule that assumes that Megan Sykes and Dan Salomon and I are to give our concluding remarks in a five-minute period at the end of the day. I think this means that we're all to be exhausted by that time. Dr. Sykes and Dr. Salomon will be serving as symposium moderators today. Thank you for doing that. I'll make a comment now and then, and if necessary, try to help us stay on schedule.

A few words about tomorrow, which will deal with updates of recent meetings and news occurrences, public comments and breakout sessions of our two SACX working groups, and a discussion of the FDA Guidance for Industry Draft Statement that all of us committee members were sent earlier and by now

surely have formulated discussion questions. We will discuss that sometime tomorrow.

Now, two words. The first word is that during the first session tomorrow under recent meetings and news updates, I want the committee to look very briefly at the suggested change in our charter. The second word is that given the work we have to do and the preparation we've made regarding our working groups, our breakout sessions are really quite brief. Nevertheless, let's begin to formulate some ideas, however brief the discussion is, about when we want these documents to be ready and what we have in mind with respect to releasing and publishing their contents, whether that be merely articles and journals or perhaps a public meeting of some kind. We'll discuss these and other issues in our plenary progress update session beginning at 11:35 tomorrow.

But on to the business at hand, our truly exciting scientific symposium program, and we are so pleased to have Dr. Megan Sykes, one of our committee members, to introduce the symposium and serve as the first moderator. Dr. Sykes?

Agenda Item: Introduction: Science Symposium on the Immunology and Applications of Xenotransplantation

DR. SYKES: Thank you very much. As Dr. Vanderpool said, today we have an outstanding group of speakers lined up to educate us on the science of xenotransplantation. As you know, we've heard a lot already about safety issues and a bit about some of the clinical trials that are ongoing, but what we haven't heard is the state of the science of xenotransplantation, where are we, what are the gaps in our knowledge, and what is needed to move this field forward, and should it move forward. So today we have enlisted a group of speakers, and I would like to thank them all in advance for coming today, to tell us first about some of the physiological interactions that affect xenotransplantation and then about the immunology of transplantation, followed this afternoon by some discussion of the latest technologies in genetic modification of donors, and also to hear about extracorporeal and bioartificial technologies and cellular transplants.

The hope is that you will come away with an understanding, first of all, of the fact that all systems are regulated and involve molecular interactions and that systems that affect the survival of a graft and of a recipient, such as the coagulation and the complement systems, as well as recognition mechanisms of the innate immune system, all have to be thought about in the context of xenotransplantation, that we have to figure out if molecular interactions that regulate processes are working between the species, and if not, figure out what we can do to make them work. And this of course is one of the great advantages of being able to genetically modify our porcine donors, and this is one of the reasons for the excitement about the proofs of principle that have recently been generated in pigs.

As far as immunology goes, this is a very special area. The immunology of xenograft rejection involves not only the adaptive immune system, but also the innate immune system, and you'll learn today about some of the carbohydrate recognition mechanisms involved in innate immunity and how those could impact on xenotransplantation. I hope that you'll come away at the end of the day with a feeling for what we do know and also for what we don't know, what we need to think about in order to make xenotransplantation work. And perhaps in the end you'll share my optimism based on the developments in porcine gene technology that we can, with an infusion of proper effort and personnel, perhaps overcome these obstacles. Thank you very much.

I'll now begin by introducing the first speaker, who's Dr. Agustin Dalmasso, an expert in the field of complement physiology and its role in xenotransplantation, from the University of Minnesota. Dr. Dalmasso?

Agenda Item: Complement in Xenograft Injury: Current Status and Unresolved Issues

DR. DALMASSO: Good morning. Thank you very much, Dr. Sykes. I am delighted to be able to participate in this very important symposium. I will be talking primarily about the role of complement in xenograft injury as it relates to primate models of xenotransplantation. All forms of xenotransplantation injury are in one way or another related to the complement system except possibly cellular rejection. In hyperacute rejection the role of complement is essential. Antibody binds to the vasculature of the graft, and the complement is activated. Rejection takes place in a matter of minutes or a few hours. If complement is inhibited, hyperacute rejection does not occur, however, in a few days another process that will be discussed later on, acute vascular rejection, becomes very important, and it results in the loss of the graft, and here complement may also play a role, although it is not essential.

Now, it's important to have an overview of the functions of the complement system. The complement system is a very complex set of proteins that participate in innate as well as in acquired immunity, and these have the major functions enhancement of antibody production, solubilization and removal of immune complexes, extremely important in the clearance of anti-Gal program cell death, even in the physiological conditions. Those cells need to be eliminated, and complement plays a major role in doing that. It's also very important in the fight of recipient against microorganisms, and finally it's a very strong promoter of inflammation. And it is through this promotion of inflammation that complement causes injury to xenografts.

Now, this is a very basic organization of the complement system, the three pathways of activations that converge at the level of C3, activating C3, and then forming the so-called membrane attack complex. This is the kind of slide that I like to use when I need to talk about complement, but I really do not want to get into it. It's a tremendous oversimplification, so I will have to give things a little bit more complicated, and I apologize for this this early in the morning. But nonetheless, the complement system is composed of more than 35 proteins actually, and here I only want to have like two or three aspects. One is the classical pathway. The first component or complement called C1 is really composed of a complex of several proteins designated as C1q, C1r and C1s. I will talk more about the lectin pathway and these other elements of the pathway, but right now I wish to highlight the receptors on the cell membranes of many cells, especially inflammatory cells and blood cells, endothelium, that contain proteins that function as receptors for the activated products of the complement system. So they are receptors for C1q, for C4, for C3, for C3a, C5a and so on.

Now, this is getting a little bit more complicated, but I only want to again indicate that the complement system can be divided into the activation path with the three pathways, classical, lectin and alternative, then the generation of two enzymes that I call three convertases because they act on C3, producing the activation of C3 or C5, resulting in activation of C5. And the final product of all this is the generation of this membrane attack complex. Concomitant with all these processes that really are based on proteolysis, or fragmentation of proteins, by the preceding complement in the sequence of reactions, concomitant with this is the generation of fragments that have biological activity, C3a, C5a, C3b, and the MAC, or membrane attack complex or complement.

Now, such a complicated system that has so much biological activity obviously is going to need to be regulated very tightly, and this slide adds the regulators. The regulators are groups of proteins that are either in the plasma or membranes, and they act at three very distinct sites of the complement sequence, the C1 inhibitor that blocks activation of C1 of the classical pathway as well as the lectin pathway, then there are these groups of inhibitors that together or individually act on the C3 convertases and the C5 convertases -- essentially they block activation of C3 and C5 -- and finally, other proteins, such as CD59, that block the membrane attack complex. I'd like to mention here this pathway with some emphasis because it is somewhat of more recent recognition than the others. And what takes place is that in plasma

we have certain proteins that are lectins. They bind carbohydrate on the surface of the bacteria or other cells, and one such of those proteins is a mannose-binding lectin that binds primarily mannose, but there are other proteins such as ficolins -- there are apparently many proteins that are able to bind sugars and certain specific sugars and then activate complement, bypassing C1, and entering into these enzymes, into C4 and C2, and then activation of C3.

Now, once complement has been activated, various fragments are generated, so these products and many others are formed. They can act on the endothelial cells of the xenograft as well as in the recipient blood cells such that monocytes or lymphocytes or platelets primarily can be activated by complement, and in turn, affect the function of the xenograft. And I will not get into all the things that these fragments do at this point because of lack of time, but I wish to just point out that in addition to C1q and C3bi, there is C3a and C5a that are extremely powerful for inflammatory activating substances on the endothelial cells of the graft, but also they will activate the macrophages and neutrophils and the platelets, generating inflammation as well as coagulation in the vessel of the xenograft. Finally, the C5b-9, or membrane attack complex, does the same thing. It acts on many cells, endothelial cells and blood cells, promoting inflammation as well as coagulation.

Now, several years ago, in fact, the very first recognition that complement was important in xenotransplantation is relatively old. That thing is about 36 or 37 years old when Robert Nelson in Miami and Henry Gewurz of Minnesota isolated cobra venom factor, which is a substance from cobra venom that kills C3. And so what they did, they gave some of this cobra venom factor to dogs I believe, and then pulled the graft of another species, and they showed the rejection, instead of occurring in one hour, occurred in 15 or 20 hours. So that was the first demonstration that complement played an important role in hyperacute rejection. But 10 or 12 years ago when we got into this area, we thought -- everybody thought -- that it would be important to take advantage of physiological inhibitors of complement to see if we could make some headway in regards to xenotransplantation. Now we have other tools that were not available 35 years ago. So C1 inhibitor could be used for that purpose as well as some of these membrane-associated inhibitors, such as DAF and MCP and CD59. And this is what has been done. I think I will skip this one.

Especially the membrane-associated complement inhibitors, DAF, or decay-accelerating factor, MCP, or membrane cofactor protein, and CD59, that act at C3 or at the level of the MAC have been employed. And this is simply a diagram that represents the protein structure of these molecules. They are all linked to the membrane, and one property of these proteins is that they tend to block complement of the same species much better than complement from a different species. So we thought that for protection of the pig organ, it will be necessary to create transgenic pigs that express human complement inhibitors because this human DAF, for example, should be able to inhibit human complement. The pig graft probably would not do that very effectively, and this species' restriction has been at the basis of all these experiments with human proteins to humanized pig organ.

Now, this field of research I'm sure is going to be summarized by others later on, so I will only say that there are still some unresolved issues regarding the use of complement inhibitors with membrane regulators. The first one, improve the expression of transgenes, I think this has been already accomplished to a very large extent. Still I think we are going to need to combine different complement regulators in pigs that have strongly reduced alpha-Gal expression, and this is something that I'm very much looking forward in the next few years to see the effects. We would need to define the extent of complement activation that takes place despite the presence of the inhibitors because these inhibitors are not working in a 100 percent manner. They will block most of the complement inhibition, but not completely. And finally, we have to reevaluate this issue of the species restriction because more recently it has been found that in reality pig inhibitors work pretty well with human complement so that perhaps one does not even need to use human complement, although at the present time the concept is that human

complement inhibitors are better than pig complement inhibitors. We may get in more detail to this in the discussion.

Now, there are also soluble inhibitors. Why even consider soluble inhibitors when membrane inhibitors work so well? Well, the reason is that there may be special circumstances where it might be advantageous temporarily, for a few days perhaps, to use some of these soluble complement inhibitors. And I will just mention a few of them. C1 inhibitor is very interesting because it blocks not only the classical pathway, but also the lectin pathway. However, it does not block binding of C1q, and this is important to know. C1q inhibitors are being developed, and this is of great interest because C1q may be involved in acute vascular rejection. However, if an antagonist to C1q is developed, it would be important to preserve those functions of C1q that are required for elimination of apoptotic cells and protection from autoimmune diseases, a very difficult task. Perhaps the best known soluble complement inhibitor is TP10 or soluble CR1 that has been extensively used experimentally and in clinical trials. Other inhibitors or antagonist receptors, such as C3a receptor and C5a receptor antagonist, are being developed. We have had some experience working with this.

This is a very old experiment that Jeff Platt and I did to show that there's a synergy between C1 inhibitor and Heparin in preventing the position of complement proteins on pig endothelium when incubated with human serum. This is really something that is remarkable, but it hasn't been used. This is another inhibitor that was developed by Grace Yeh. It's called CAB2. It's a molecule derived from DAF and MCP together, and we have tested this inhibitor in vivo in pig-to-rhesus monkeys' heart survival experiments, and we see that the use of this inhibitor prolongs the life of the graft from less than one hour to four days. And we look for evidence of complement or lack of complement activation and reducing C3a in the plasma so that during the four days the level of C3a would not elevate. When we looked at another indicator of complement activation, the level of plasma, or C5b-9, we demonstrated that even in the animals that receive multiple doses of CAB2, at the time of rejection there was a slight elevation of this C5b-9 complex, the important point being that it is not only important to study deposition in the tissues of complement components, but also to make sure in the plasma on a frequent basis the presence of this activation, the fragments of complement.

Now, the use of complement inhibitors I believe cannot be done for a very, very long time, and the reason is that individuals who have -- humans that have deficiencies of one of these proteins, most of them have a great severity to being in trouble with -- for example, patients deficient in C3 will have pyogenic infections. Patients deficient in the early components, immune complex disease, may see infections and so on. So whether chronic systemic inhibition of complement activation is a good idea, I think not because of the reason I mentioned. There may be some inhibitors, such as the antagonist of C5a receptor, that it may be quite all right to use those because the pro-inflammatory effect of C5a is not essential for the life of an organism or a human. However, C5a has functions that are very important in relation to regulation of IL-12 production and TH1 responses, so they still need further work.

Of course, there are unresolved issues with soluble complement inhibitors. I would very much like to see C1 inhibitor tested in vivo in pig to primates, and then I think that we need the development of C1q inhibitors, for example, and C5a receptor antagonists that are useful as drugs to give to patients by mouth that are small, that are nontoxic, and they behave pharmacologically as pharmacological agents do, and we don't have those inhibitors.

Now, I wish to talk briefly about another phase of the graft. Here instead of inhibiting complement activation to prevent injury to the graft, we would modify the graft so that the effects of complement and of other immunological effectors of injury to the graft do not occur. Accommodation has been defined as the ability of a graft to survive in the presence of antibody and complement. We used to say that to induce accommodation in primates, it was going to be a similar difficulty, if not impossible, but recently

Lin and Jeff Platt have demonstrated that accommodation can be achieved in pig-to-baboon heart transplantation, of course with intense immunoabsorption of anti-Gal and based on immunosuppression. Complement does not seem to play a role in induction of accommodation in rodents, but we don't know what happens in primates. And protection from complement injury is a useful indicator of accommodation. It's not in itself accommodation. So when one does cyto protection studies in vitro, one should look at protection from complement, but also from apoptosis, oxygen radicals, and most importantly, cell-mediated injury.

And here, for example, we've shown that alpha ligation under certain conditions would induce protection from killing or cytotoxicity by complement when this lectin that binds alpha-Gal is used. And also there is protection against induction of apoptosis and very good protection against killing by oxidative damage in the form of hydrogen peroxide. And we have not yet developed this and made this toxicity here.

But there are major unresolved issues related to accommodation, and I think this is a field that deserves a lot of attention. We need to have methods for inducing accommodation in pigs to primates that do not compromise the recipient. Then we need to study to understand the mechanism of induction and maintenance accommodation in pig to primate. In rodents this is extremely well known, but not in pig to primates. Additional unresolved issues related to complement refer to the role of early components of the classical pathway, especially C1q, and the lectin pathway in ischemia/reperfusion injury and in acute vascular rejection. We need to know whether the activated endothelium is stable itself to activate complement by either pathway and establish if this activation is sufficient to cause significant injury. Additional unresolved issues refer also to whether anti-pig antibodies of specificities other than anti-Gal activate complement, and what is their significance. And this is going to become very important now that donors that express no alpha-Gal are going to become available. What happens to that particular surface? And I'm sure we'll hear more about that later on. All the in vivo studies in primates should include a careful assessment of complement activation, and in most cases it's done, but both tissue deposition and also plasma level of activation fragments should be measured.

There are certain issues related to complement that we really do not know if they are going to come up and what they are going to be, but we can learn from the experience with allotransplantation. It has been recognized in recent years that in allograft injury antibody and complement may be implicated more often than assumed in acute and in chronic rejection. A specific example is the C4d deposition in peritubular capillaries of the kidney where it was found more frequent, C4d deposition, than IgG and C3 deposition, and in the absence of serum antibodies sometimes, indicating that perhaps known antibody-mediated activation is responsible in some of those cases of graft injury. So the unanticipated issues on the role of complement in xenotransplantation may not become clear until the acute vascular rejection barrier has overcome these type of problems.

In conclusion, significant advances have been made in understanding the contribution of complement activation as part of the barriers for successful pig-to-human xenotransplantation, also in understanding the mechanism of complement activation, and in developing strategies to prevent complement activation by expressing membrane complement regulators -- I think that this has been one of the major advances in the field of xenotransplantation -- by reducing the antigenicity of the porcine endothelium and reducing the preexisting as well as elicited antibodies in the recipient. However, future studies are essential to fill the gaps that still exist in understanding the mechanism involved, continue to evaluate the role of complement inhibition, in combination with donors that express reduced antigens as well as in organs that exhibit induced accommodation, in recipients with reduced preexisting and induced antibodies. We also need to know about more effective and appropriate ways of inhibiting complement with membrane and with soluble inhibitors. And finally, we need to establish very clearly that a particular strategy for complement inhibition is not harmful to the recipient or to the graft. It's to say we cannot create trying to help the graft and hurt the patient by not using the proper therapeutic strategy.

And my final comments are, number one, to emphasize the obvious. Antibody-mediated complement activation is not really a complement problem. It is an antibody problem. It's not a problem for me. It's a problem for the antibody people. However, for me there remains the problem that if the antibody problem is solved in the case of xenotransplantation, complement may still be activated through antibody-independent mechanisms, for example, by the lectin pathway or even the classical pathway, direct activation without antibody.

Now, a completely different issue is simply mentioned here. Complement is a link between natural and adaptive immunity, and as such it may be involved in xenotransplantation through mechanisms totally different than what I have been talking about so far. They may be involved in xenotransplantation through control of cellular immunity, through control of the antibody response, as well as in tolerance induction. Thank you very much for your attention.

DR. SYKES: Thank you very much, Dr. Dalmasso, for a very lucid and comprehensive presentation. We've organized the schedule today so that we have five minutes after each presentation for questions immediately related to the points of clarification, and at the end of the morning session, and then later at the end of the afternoon session, we will have a panel discussion where there's time for more in-depth discussion. Does anybody have a question, a point of clarification, for Dr. Dalmasso?

DR. SALOMON: Just quickly one of the things I was struck by in your data with the CAB2 inhibitor was that despite continued administration of it, there was an escape, so you had this great result that you prolonged graft survivals dramatically, but then it escaped despite administration. And then as you went on, this sort of dynamic between accommodation and successful inhibition of complement really turns out to be a key. You did a great job of showing how critical that will be. So the question I had for you is can you successfully inhibit complement long enough to allow accommodation, and on the other side of the coin, is it possible that inhibiting complement activation will prevent accommodation?

DR. DALMASSO: So far I believe that complement has very little to do with accommodation. Complement is in the way of accommodation in some models, but the key issue -- and Jeff Platt may speak to that -- the key issue in accommodation is to remove the antibodies, not to remove complement. In fact, in the ABO incompatible allotransplantation accommodation phenomena, their complement is not inhibited. The only thing that is done is reduction in antibody levels and maintaining for a sufficient period of time. So I'm not saying that complement is not involved. Complement might be involved, but we don't know. But what we know is that the important issue is the antibody. Complement can stay there if there is no antibody.

DR. SYKES: You've alluded to the lectin-induced pathway of complement activation. Of course this is of considerable interest considering all the carbohydrate differences between porcine and human cells. Do you know of any direct evidence that the carbohydrate expression pattern on porcine cells activates this lectin pathway?

DR. DALMASSO: I'm not aware of any -- I think there must be one study, but I don't know the study very well. I am more familiar with the study from Greg Stahl on oxygen-stressed endothelial cells, but these were human endothelial cells. When these cells are deprived of oxygen for a long period time, and then reoxygenated again, then the endothelium changes such that certain elements of the endothelium become now able to bind the mannose-binding lectin and activate complement. So I think the thing is that the lectin pathway is -- there are many substances that can activate the lectin pathway, not only MBL, but also another group of substances called the ficolins. So it is very likely that they are going to be very important perhaps in a more subtle type of injury than the one that we get through the classical mechanism complement activation.

DR. MENDEZ: A follow-up on Dan's question on accommodation. When we started crossing the major ABO blood barriers in the '80s and early '90s, we found a perplexing situation in which we would lower the antibodies by plasma freezes and the splenectomies and transplant the patients when they had low antibody titers. If their antibody titers raised significantly in the first three weeks, we had hyperacute rejections. However, after three weeks the antibody titers could go through the ceiling, and there would be no change in the graft function. The graft functioned completely well. And we were perplexed as to whether or not this was some sort of receptor site blockage or whether or not the actual antibodies that were being formed after three weeks were different than the antibodies that were formed before, although they were all IgM type of antibodies because this was a primitive type of analysis. What would be your views on that? Is it a different type of antibody that's forming in accommodation or is it something to do with receptor site bindings or neither of those?

DR. SYKES: I'm going to ask you to make this response rather short, even though it's a long question, because we are running out of time. We can continue later.

DR. DALMASSO: I think that there are possibilities that we don't know the answer. It may also be related to activation of protected mechanisms that are occurring at the time when you observed the rejection. But of course, nowadays the lower level of antibody is maintained. There is an effort made to maintain the levels lower beyond that critical period that you mentioned of two or three weeks.

DR. SYKES: Thank you very much.

DR. VANDERPOOL: Megan, could I make one point from the Chair? I hope from time to time that the science members of the committee and of course the speakers will put comments into perspective for nonscience people. You know, it's difficult for a lot of people to even know what the complement system is, and then we hear about complement, and we hear about accommodation, and we hear about antibodies. And this can be really confusing for nonscientific people. I think I got a good bit out of the presentation, such as the complement system is very complex and is being worked on significantly, and changes are occurring, but I hope my level of comprehension can exceed that as we go along.

DR. SYKES: The next speaker is Dr. Simon Robson from Harvard Medical School. He is going to talk about disordered clotting and heightened platelet activation in xenotransplantation. he coagulation system is another very complex system, unfortunately, that has many parallels with the complement system in being tightly regulated by a variety of molecular interactions, and the two pathways, in fact, intersect at a number of points. And we've asked Dr. Robson to give us an overview of this system and its role in xenotransplantation. Dr. Robson.

Agenda Item: Disordered Clotting and Heightened Platelet Activation in Xenotransplantation

DR. ROBSON: Thank you, Dr. Sykes. Thanks, Dr. Salomon, for the invitation to present here. Mr. Chairman, ladies and gentlemen, good morning. My talk will address the disordered regulation of blood clotting and platelet activation within xenografts. I'll touch on the mechanisms how this occurs, what the implications are, and I'll make one or two suggestions as to how we may address this.

Essentially, where I start with are mechanisms of coagulation, platelet activation, these are small little fragments of cells which are circulating in the blood which initiate clotting. Once you have a clot, you have to break it down, that is called fibrinolysis, so I'm going to touch on these mechanisms and how they actually pertain to xenotransplantation, where the grafts are subject to a really intense clotting procedures. I will touch on problems that we have when we infuse cells. These are xenogeneic progenitor cells which

cause intravascular problems, clotting to vital organs such as kidneys and brain. I'll then touch on the more classic features that we've seen when we transplant a xenograft to a vascularized xenograft where the blood vessels are from the actual pig. And these are not like the actual teflon we have in our blood vessels normally, they are very, very sticky. They cause clotting, and they cause the grafts to actually succumb very rapidly.

I'll touch on what the pathogenic factors are, whether the antibodies are implicated in this, the complements involved, possibly the rotaviruses, and then the cross-species molecular incompatibilities that Gus has already alluded to with regard to complement, the fact that regulators of coagulation in our blood vessels don't work very well with pig proteins and vice versa. The implications for this, certainly a delay in the clinical studies and so forth until this problem is worked out, and then I'll touch on what we think of the important anticoagulant antithrombotic and antifibrinolytic interventions, and what the long-term options are.

My comments are strictly applying to discordant combination, where you are transplanting a pig organ into a baboon or primate. In this discordant combination the coagulation is much more profound than what you would see in a concordant combination. Certainly here you can see a photomicrograph of a pig-to-baboon cardiac graft undergoing hyperacute rejection. And you can see over here extensive hemorrhage into the tissues where the blood vessels have just basically been demolished, the endothelial cells, the lining of the vessels have just retracted. The blood is basically just gushing into the tissues. You see clot formation, which is a thrombus, a pathological thrombus, and you see lots of platelets sticking to the endothelium.

Now, as Dr. Dalmasso said these are complement dependent responses. This causes hyperacute rejection occurring in minutes to hours. The problem I am going to allude to mainly is the current concern that you can get persistent blood clotting responses in xenografts in the apparent absence of intense complement activity. And the main components of this, obviously the coagulation proteins, which are circulating in the blood which form this fibrin clot. Fibrinogen is a protein in our blood circulating in very high concentrations. It's soluble, it's acted on by enzymes, it becomes fibrin and forms this clot. Involved in this are the endothelial cells, which initiate the clotting and platelets, these fragments of cells which circulate in our blood and initiate the actual thrombus.

Red cells get tied up in the thrombus as well, as do white blood cells. So the concern is that this occurs despite the absence of complement. Now I'm going to have to touch on some mechanisms of hemostasis and clotting. It's a very complicated problem how this actually occurs. We all have to have primary hemostasis. If we cut ourselves, we have to actually stop that bleeding, otherwise we'd bleed to death. This is the primary hemostatic mechanism with platelet adhesion leading to platelet aggregation and a platelet plug, this hemostatic plug. It ties in very much with secondary hemostasis, which is what happens when you get coagulation proteins which are acting together in a very complex cascade very similar to the complement pathway. This is initiated by something called tissue factor, excuse me. Tissue factor is expressed by tissues, except the lining of the blood vessels endothelium. Only when you activate endothelium and damage it, only then does it express tissue factor. The tissue factor initiates coagulation you form at the end of all this pathway thrombin from prothrombin. Thrombin is the enzymes which catalyzes the formation of fibrin and you form the hemostatic plug.

So what I'd like to say is that you have platelet and coagulation factor activation, you have lots of exciting biochemistry and physiology occurring, and then you get this clot. This doesn't project very well. This is what basically we've taught in medical school, how clotting occurs. You have an intrinsic pathway over here, which basically occurs if you take blood and you put it in a test tube, its intrinsic properties of the blood, and you get clotting. This is also associated with an extrinsic pathway, which occurs in the blood vessel. So the extrinsic pathway for a long time was thought to be the poor relative, but the major

problems always occurred in the intrinsic pathway, and the reason for that is the disease called hemophilia, with Factor VIII deficiency, which you may have heard of, you know, the actual Russian royal family and Queen Victoria and so forth. A lot of interest in coagulation was derived by the interest in hemophilia, Factor VIII deficiency. It now appears that most of the actual clotting which occurs in our bodies in vivo, not in a test tube, is dependent on the extrinsic pathway and tissue factor. The only reason that Factor VIII is involved is it takes part in an amplification loop causing much more factor X, and then you get the thrombin generation.

Platelets are involved in this process as well, and obviously when you have a clot formation, you don't want it to sort of suddenly progress through your body like in that science fiction movie "The Andromeda Strain," where the virus attacks humans and the endothelial cells all get activated, and they basically clotted to death within minutes. So you need a process which actually stops the clotting, and you need a process to remove clot. That is called fibrinolysis. That is also a very complicated pathway.

What I want to address are the two major important pathways under coagulation. You start with tissue factor, over here. You start with a tissue factor, you bind circulating clotting factors, you activate factor X, you generate thrombin, generate fibrin. This is then acted on by the fibrinolytic pathways and plasma factors and then the clotting processes are brought to a standstill.

The important anticoagulants in your blood and the endothelial cells are something called tissue factor pathway inhibitor, which inhibits tissue factor, and thrombomodulin, a modulator of thrombosis, which inactivates the actual clotting factors V and VIII and basically brings this whole process to a standstill.

And the reason I'm spending so much time on this and so much detail on telling you about tissue factor pathway inhibitor and thrombomodulin, as I said, don't seem to work very well in the pig-to-primate combination. When you transplant a pig graft, it has tissue factor pathway inhibitor on its endothelial surface, it has thrombomodulin on its surface, and work from our lab and Dr. Jeffrey Platt's lab and other laboratories have shown there is a functional incompatibility, that these anticoagulants do not work well across species barriers.

So you are transplanting essentially a thrombophilic sticky vasculature into your recipient. And you can see this when you look at the actual vascularized xenograft rejection, and you are measuring thrombin activation. What we see over here on the vertical axis are markers of thrombin activation. This is F1 plus 2, and this is TAT. They're just surrogate markers of the thrombin being generated. And over time following the transplant you see initial blips, blips at the time of the initial procedure, which is obviously your clotting occurring at the time of the surgical procedure, and when you get rejection occurring, you get massive increases in thrombin generation, and that causes problems, because you get a process then called consumptive coagulopathy, otherwise termed in some circles disseminated intravascular coagulation. So you are getting a process where the coagulation pathways get totally out of control, and you lead to extensive fibrin deposition and activation of clotting within the actual graft.

That is the thrombin generation, so there is a thrombin problem when you transplant a xenograft. The other problem is up-regulation of tissue factor, and you can see over here that the actual porcine xenograft on the endothelium, which is supposed to be clear of tissue factor, that once you get binding of antibody, you activate the endothelium, you up-regulate tissue factor, and it's as though your blood is just seeing basically a big wound, and it just starts activating, and clotting starts developing.

I just want to touch on these blood platelets. These are what they look like when they are circulating through our body. They are small fragments of cells. When they activate, they stick to the endothelium. They also stick to the gaps the endothelium leaves on the subendothelial matrix, and they cause platelet plugs.

Now, platelet activation, again, is a very complicated pathway, what you need to take away from this presentation is that platelets are rolling on our endothelium the whole time. They go into the actual peripheries of the blood vessel, and they start rolling, as you can see over here, and they roll on factors like selectins, which are carbohydrate- recognizing structures, which are going to be speaking about in the next lecture, and also the von Willebrand factor. Now von Willebrand factor in a protein in the subendothelial matrix. Abnormalities in it can cause a form of hemophilia. Von Willebrand factor that is present in the pig vessels is very, very sticky, so the platelet would tend to roll much more avidly on this, and potentially have much more options for activation. That is the first phase of activation of platelets. They are rolling around the blood vessels. They get much more slowly rolling at areas of vascular injury.

The type 2 of activation response of the platelet is where they form the plug, and here what happens is they stick down to the von Willebrand factor. They stick down to collagen or other adhesion proteins in the subendothelial matrix. A factor called ADP or ATP are released. These are the basically energy currency of cells which are present inside cells in high concentrations. They are released following cellular damage. They are released following platelet activation, and they cause a major activation response, and the platelet plug develops.

In the absence of ADP, you will not get a platelet plug. That is a very important point, and it's only been recognized the last few years. All platelet activation is dependent on ADP, the final platelet plug. You need high concentrations of ADP to activate the platelet.

And why is that important? Because on all our blood vessels we have high levels of enzymes which remove ADP. And we were able to show that this major enzyme doing this was something called CD39. And what this enzyme does is it removes ADP, which otherwise is initiating platelet clot, and forms adenosine, which actually prevents platelets from clotting. So this enzyme is uniquely situated to convert an environment where you have high levels of ADP to one where you have high levels of adenosine, and therefore convert a prothrombotic environment into an antithrombotic environment.

The problem is in a xenograft, this enzyme is very subject to oxidant stress, and once you get hyperacute rejection of a pig xenograft, the enzyme activity is lost. And this is a pig kidney showing the actual loss of the enzyme in the glomerulus with the hyperacute rejection. So you can imagine you've already got a sticky endothelium transplanting into the actual baboon. You get hyperacute rejection, you get ischemic reperfusion injury, you lose the enzyme activity, platelets become much more able to activate and form this platelet plug.

Fibrinolysis, I'm not going to touch much on. Most of the work is being done by Jeff Platt's lab. Suffice to say there seem to be factors which would actually impede fibrinolysis, so not only are you putting down more clots, not only are you activating more platelets, but you are also preventing aspects of fibrinolysis. But again I will not address that today.

The models that we've been looking at with Dr. Neiman Sykes' lab and Dr. David Sykes are basically the baboon-to-pig with tolerance induction. It's a very complicated model which involves splenectomy, irradiation, immunosuppression, and induction of tolerance by infusion of porcine cells, plus or minus the pig xenograft.

Experimental groups are being looked at all with the same conditioning, with alloconcordant cells, autologous cells. And in these first two groups, the intravascular coagulation, the thrombotic complications do not occur. They only occur one we start transplanting porcine xenogeneic cells, either the Porcine progenitor cells, or transplanting the porcine xenografts, or doing both together. And in those last three groups, this is published work. We have major thrombotic complications.

Let's touch first on what happens when you infuse cells. Again, a complicated regime removing complement, or removing antibody, giving immunosuppression, and you can see you actually -- even the actual porcine cells themselves, in the absence of the actual tolerizing regime, you are giving the pig progenitor cells over here, you are monitoring the platelet count on the vertical axis, and you can see major drops in the platelet count at the time of infusion of the cells. And, again, you repeat it over here.

If you give whole body irradiation and the full tolerance-inducing regime, plus the porcine cells, you can see there are really profound drops in the platelet count following the infusion of cells seen over here, so major thrombocytopenia. The problem is what is happening to these platelets? And what is happening to them is they are actually getting stuck onto the actual progenitor cells which are being infused.

This is a normal smear of a baboon, blood smear, before the infusion of precursor cells. And this is after infusion of the cells where you are seeing fragments, and you start seeing platelet clumps. You see platelet aggregates forming, very large aggregate forming in the actual bloodstream. And obviously you can't circulate particles, big particles in your blood like that, particularly of platelets, because platelets are just full of clotting factors. They are full of ADP, they really are noxious things to have circulating around in an aggregated state. What they do is they stick in blood vessels. And they stick in the blood vessels of a kidney, and they stick in the blood vessels of the brain, and they cause a process called thrombotic microangiopathy, where the animals become comatose. They develop renal failure. They develop bleeding disorders, and this is just a picture of histology of a kidney of one of these baboons obtained for platelets. And you can see massive deposition of platelets within the kidneys. And what has happened to this animal, it's being given pig cells, they've caused a clotting inside the bloodstream, and they basically are causing damage to the peripheral tissues. This obviously precludes the use of this, and it also means that the actual pig cell is not going to where they need to go into the bone marrow. They are already being acted on by platelets, and basically sequestered in the bloodstream.

So we asked a question: Do we get direct interaction of pig cells and platelets? And does this result in the thrombotic microangiopathy? So we mixed together pig cells and baboon platelets in vitro and did some aggregometry. This is just looking at light absorbance over time adding pig cells at high concentrations basically causes a platelet plug to occur in a test tube, and you get increased light absorption. So in these aggregometry profiles, the addition of pig cells causes platelet activation in vitro.

We did some immunohistochemistry of these, and you can see that there are extensive platelet aggregates in the brown staining for P-selectin mixed in with these pig cells. And this is what is directly happening in vivo, and the main factors causing this appears to be P-selectin on the platelets binding to carbohydrates on the pig cells.

We are able to inhibit this by using essentially the GPIIB blocker. This is an adhesion protein on platelets, which causes the platelet plug to develop, and by inhibiting this, we are able to actually block the aggregation of platelets.

Now these porcine cells not only have effects on platelets, but also have direct effects on human and baboon endothelial cells. Again, they directly activate endothelial cells by cell-to-cell contact. They activate all the factors which are important in binding leukocytes and activating of the endothelial cells. And it seems that the mononuclear cell macrophage population are important in this context. So if one could further purify Porcine cell population to pure progenitor cells, one might have less effect on the endothelial cell activation. That remains to be seen.

With regard to the xenografts, per se, when you transplant these xenografts again at the time of the actual organ transplant, you have changed the fibrinogen levels. When you get the rejection process occurring,

you get drops in the fibrinogen levels with a consumption of clotting factors. The fibrinogen is dropping in the blood because it's all being deposited onto the actual xenograft. The animals start bleeding because they are clotting only in one place, and they are consuming the factor which prevents them from clotting elsewhere. And if you take the grafts out, you see rapid recovery of the fibrinogen levels, and the animal's clotting problems basically resolve.

If you give porcine grafts, plus cells, you get really exacerbated coagulopathy, you get both that microangiopathic change, plus the DIC, and several of these animals have succumbed and only can be rescued by removal of the graft. Interestingly, when you take the grafts out, it's much more impressive the coagulation problems with a renal xenograft than with a cardiac xenograft. And often you only see very mild features of antibody-mediated rejection with interstitial hemorrhage. And in some instances the grafts almost look fairly normal. So you are getting the coagulation process developing in the presence of really mild antibody mediated rejection. Sometimes of course you get full-blown acute vascular rejection with a full DIC. The antibodies themselves may be mediators of vascular inflammation. We have been able to show that Gal reactive epitopes and antibodies directed at Gal are important in cellular activation responses. We have also shown induction of procoagulant responses on endothelial cells in vitro, induced by xenoreactive antibodies. And it's important to say here that it's not just natural antibodies with complement that do this, but elicited xenoreactive antibodies directed at both Gal and non-Gal epitopes are able to activate the endothelium. And that is in the absence of complement.

So complement can do it, sure. Elicited antibodies possibly directed against non-Gal, that is still preliminary data, are able to induce coagulation. The other factors are porcine viruses. We have only recently recognized these as a problem, and we've done some work now showing that porcine cytomegalovirus can induce cellular activation and tissue factor up-regulation on Porcine E, C and B2. Whether PERV are important to these endogenous retroviruses remains to be seen. But I think once you got rid of the antibody problems, you might have to address the virus problems as another factor important for disordered thromboregulation within the graft.

Now the final part of my presentation over the next two to three minutes will address some of these molecular incompatibilities. And I'm sorry this is a little bit complicated, but remember I told you tissue factor is important in initiating clotting. Thrombin is important at the bottom of the cascade to cause the clot.

Now, the tissue factor pathway inhibitor on the pig endothelial cells doesn't work very well when you start circulating baboon or human proteins across it. So, for example, the factor 10A, that middle point, is not inactivated by the actual tissue factor pathway inhibitor. So already at the beginning of the cascade, you have a problem where you are not actually effectively inhibiting the clotting. The other side is the thrombomodulin which again is expressed on endothelium, doesn't work very well with the human or baboon circulating anticoagulants, and you are not able to bind the protein C. You are not able to inactivate the thrombin and the precursors. The other problem with the von Willebrand factor directly acting on the platelets, it's much more sticky, the platelets are much more avidly bound to the endothelium and subendothelial matrix. So these are really analogous in their regulation of complement. Their significance is not fully determined. I mean if you are actually showing an incompatibility of 80 percent, and you are able to up-regulate these, you may be able to retain some degree of coagulation. But obviously if you are able to over-express these, you may be able to block some of the coagulation problems that we see.

This is a tissue factor pathway inhibitor. It's expressed on the endothelium. It can block a tissue factor expressed by the subendothelial matrix also by monocytes, and initiates obviously the common pathway of coagulation.

In the pig, there seems to be a truncation. You lose aspects of the C terminus, and that prevents it interacting adequately with factor 10A, so we have a molecular mechanism for that. The porcine thrombomodulin is not able to avidly to generate protein C which inactivates the factors V and VIII, so again there are some functional immunological properties of porcine thrombomodulin which explain its incompatibility with the human proteins.

With regard to the von Willebrand factor, it's important to note the von Willebrand factor GPIb interaction only occurs in our blood vessels when the platelets are rolling. And the porcine von Willebrand factor we've been able to show spontaneously reacts with the human GPIb. You don't need that sheer stress, you don't need rolling, the protein is already ready to go and to bind platelets, so it's much more sticky for the actual GPIb. So the implications of this are, at the very least, a delay in initiation of studies until the significance of some of these perturbations in thrombin regulation are fully determined. Also we have noted that systemic abnormalities in coagulation appear more associated with a renal xenograft and possibly we could address that in the actual discussion. Certainly we don't see the same problems with the cardiac xenografts to the same extent.

But I think the message I would like you to take home is that maybe transplanting a thrombophilic graft vasculature, so this graft is just waiting for mischief. Once you activate it with viruses or a little bit of rejection, it's much more prone to develop thrombosis. And this may have long-term sequelae and limitations in engraftment and organ transplantation.

There are multiple treatment options. Obviously a lot of the movement in this area has come from cardiovascular diseases. But we could use a direct antagonist of thrombin, we can use recombinant antithrombins. We can use activated protein C. We can use other anticoagulants to infuse. We can use platelet antagonists. We've done all those, and they do make a difference, but they, you know, whether the difference is two or three days or so remains to be fully determined. My view is that we need to develop fully soluble monomeric forms of CD39, and my laboratory is working actively on that.

Fibrinolytic modalities remain under review. Other options include gene therapy and generation of transgenic animals that we are actively also pursuing that would over-express human anticoagulants. And we have chosen thrombomodulin and tissue factor pathway inhibitor. And for the regulatory factors, we have chosen CD39. So by that approach with tissue factor pathway inhibitor and thrombomodulin, we are cutting coagulation. With CD39 we are covering platelet activation. And we have not yet decided whether to also cover fibrinolysis, because we thought that might lead to a greater propensity for these animals to hemorrhage.

For the immediate future, we are going to be using purified xenogeneic precursor cell infusions, modifying these with genetic gene therapeutic approaches to allow these cells to over-express these anticoagulants and thromboregulatory factors. We are looking at preventing some of the xenoreactive antibody induction with immunosuppression and tolerance that Megan Sykes will be addressing.

Viral-mediated cellular activation we are addressing in our laboratory because this obviously may be a very important pathway that we would need to address with antiviral therapies. Our main hope is that with transgenesis and some novel antithrombotic therapies, that we may be able to overcome this. So thank you very much for your attention.

DR. SYKES: Thank you very much, Dr. Robson, for again taking a very difficult and complex area, simplifying it for us, and showing so elegantly where some of the incompatibilities are between porcine and human regulatory interactions, and how this affects a large animal, xenotransplantation model, really highlighting its importance. And, finally, for pointing out to us some of the potential solutions to these incompatibilities. Before we go on to questions, I think Dr. Vanderpool would like to make a comment.

DR. VANDERPOOL: My comment is that in saying earlier that I hoped the non-scientific members would be able to understand what is going on. I think when we joined this committee we probably should have had an informed consent statement saying "I accept the responsibility of coming fully up to speed on the scientific aspects of xenotransplantation." It is a challenge for us, but I think we have to meet the challenge, the non-scientific members. At the same time, we need comments to clarify what is going on in ordinary terms so we can get there.

The other factor that is very important is that Dr. Megan and I briefly exchanged over, is that any comments that I might have made do not suggest that the scientific members shouldn't be able to ask whatever complex questions you wish to, because we don't want to suppress the full scale knowledge and understanding of the scientific members on this committee from these excellent presentations.

DR. SYKES: Dr. Chapman.

DR. CHAPMAN: Yeah. Point of clarification. But I'm going to have to state my understanding of your key points in order to ask the clarification. Okay, if I understand what you said correctly, and it's outside of my field, every living creature like humans, for example, have things in our blood that help us clot, which is important to keep us from bleeding to death when we get cut, and things that keep us from clotting in dangerous ways that prevent strokes, which are the things you were talking about, anticoagulants.

And if I understood what you are saying, you are saying when you take the -- the anti -- When you take things out of one species, like a pig, and put it into another like a primate, the anticoagulants, the things protect against dangerous clotting, don't seem to work as well.

DR. ROBSON: Don't fully work, yes.

DR. CHAPMAN: And the things helping clot go into overdrive, resulting in dangerous things like rejection of the transplanted organ and strokes, and death of tissue, and everything. And one of your recommendations was that the mechanisms behind this, the science needs to be understood better before you proceed with a lot of clinical trials. But if I understood correctly, you also felt like there had been a lot of significant progress there, and you pointed out a number of promising approaches for the future. I wondered if you could summarize, maybe, say in your opinion, what are the three to five biggest advances in understanding, and what do they suggest in terms of future research that might solve this problem?

DR. ROBSON: I think the molecular incompatibility is potentially the one main thing. We are not going to be altering by removing antibodies, blocking complement and other immunosuppressive modalities. So it's something which is outside the current therapeutic approaches that have been developed. So even if we remove antibodies successfully, we are still going to have this thrombophilic vasculature. When I say "thrombophilic," loving to thrombus. So if you get a viral infection or something like that which causes a perturbation, which normally your system would be able to adapt to, for example you get a cytomegalovirus and wouldn't cause any major problem to us, and it's obviously the endothelium cells were fully destroyed. But in that pig xenograft, even a little bit of activation made it tip its balance, because as we are sitting here, we are clotting a little bit, and then we're blocking it and we're causing a bit of fibrinolysis. If I bang my leg against this podium, I'd get a little bit of activation of endothelial cells. I fortunately control it. I don't end up with a thrombosis in my leg.

But you add to that scenario if that was a pig graft or something, that even that little bit of injury would be enough to initiate clotting. So the approach that I would put forward to you, because you are actually

using the xenografts, we are able to modify them genetically. We can up-regulate thromboregulatory factors on that endothelial cell of the xenograft. What I would suggest we need is a multi-pronged approach. Obviously we need complement regulation, we need to drop the Gal levels and so forth. We may need something to prevent mononuclear cell infiltration, as Jeff Platt will be addressing. But for the clotting side, I think at the very least we would need a tissue factor pathway inhibitor to block the top, we would need an anti-thrombin to block the bottom. That would be -- I would love to use thrombomodulin. There are other modalities that could be used, anti-thrombin is another anticoagulant expressed on the endothelium, and seems to work fairly well across the species barriers.

So we need to prevent clotting, we need to also to prevent platelet activation. A bit more difficult, the von Willebrand factor is fairly ubiquitous in our blood vessels. You can use von Willebrand disease pigs which have deficiencies in von Willebrand factor. The animals are very difficult to breed and so forth, so one could make modifications of the von Willebrand factor. We have a crystal structure now. We know what causes the binding. My current approach is to use a factor which prevents excessive platelet activation, you allow the rolling to occur, but you prevent that platelet plug from developing by removing excessive amounts of ADP. Now whether you can do all three in one animal and not have it bleed to death or be very subject to surgical trauma and so forth remains to be seen, but that would be my current goal and opportunity to do, yeah.

DR. CHAPMAN: So, again, for the noncoagulation specialist, is it fair to translate that as saying you are suggesting that you think the most promising things might be attempts to change the surface of the organs you are putting into another species so that they don't induce this clotting as much. This is the kind of thing people are trying to do when they create transgenic pigs.

DR. ROBSON: The other reason I put some emphasis on the actual infusion of cells, is that if you put cells into the blood vessel, you know, into the baboon, you are showing the actual blood a lot of multiple small little xenografts. So by modifying those with, you know, viral modalities and so forth, we are able to show some of the efficacies of the transgenic approach.

DR. CHAPMAN: And then you're suggesting maybe you can find things that you can take out of the receiving animal that will decrease kicking off this?

DR. ROBSON: Certainly when you start using immunoabsorption to remove antibody and, you know, achieving accomodation and so forth, at the same time you are also removing clotting factors just by virtue of what you are doing. I think this is still very much speculation, what I have been showing you. I think it is important, and I think a lot of work needs to be done, and I think that time frame is probably something on the order of five years before we see how important it really is, and how best we are able to address this.

DR. SYKES: Thank you very much. We'll move on now to the next speaker, who is James Paulson from Scripps Research Institute. He is going to tell us about sugar-coded leukocytes. Now just to put this in perspective, you have heard a little bit in past meetings, or quite a bit, really, about this Gal carbohydrate moiety expressed by pigs and not humans, and many primates. Gal is a terminal sugar that is expressed on many, many proteins and lipids in the pig and other species, and you've heard about how natural antibodies in human and baboon and so on, serum, recognizes Gal, and you heard a little bit this morning from Dr. Dalmasso about how molecules that recognize carbohydrates, molecules called lectins, can activate the complement pathway.

These lectins, molecules that recognize carbohydrates, can do many other things as well. And this is an area that hasn't been well-explored really in the xenotransplantation field. And Dr. Paulson, who is an expert on carbohydrate lectin interactions, is going to educate us on some of the basic science in this area,

and relate it to xenotransplantation. Dr. Paulson.

DR. PAULSON: Thank you very much for the kind introduction. Dan Salomon, when he invited me, we have lab next door to each other, he said, "Well, we know about alpha-Gal carbohydrates, but could you tell us about what is coming up with other sugars that might be of interest to the xenotransplantation field.

Well, I am planning to talk about sugar-coded leukocytes, just to imply that sugars carry information, like other macromolecules. And of course we know that, as I just mentioned, and has been mentioned in previous talks, and is going to be talked about much more, there are xenoantigens that are sugars, most notably the Gal-alpha-1,3 Gal antigen, but there is other sugar-coded information that is of relevance to immune function, and so I will spend the last part of my talk, in fact most of the talk, talking about carbohydrate binding proteins that mediate leukocyte function, and just give a couple of examples from two major families, C type lectins involved in leukocyte trafficking, and the siglecs, which you may not have heard of, involved in receptor signaling.

If carbohydrates are the focus of this talk, and they mediate both a function and produce unique antigens, ultimately that information has to come from the DNA and the RNA, it is encoded in our genome, and so the translation from the DNA to carbohydrates comes from a class of proteins known as glycosyltransferases, which build the sugar chains. And these enzymes can be expressed differently in different tissues and different cells, and therefore give you different terminal carbohydrate structures that carry information. And with respect to pig and man, of course, these enzymes, not just glycosyltransferases, can produce carbohydrate structures in pigs that are different in man because of the expression of the enzymes.

So carbohydrates, as I've already alluded to, mediate a lot of cell surface interactions with the binding of advantageous agents, viruses, bacteria, toxin, and of course, most importantly for this group, antibody, but they also mediate cell-to-cell communication. And this was just an idea less -- or a decade ago or so, but is now quite well-developed as an idea, but still early in its -- in its evolution.

Focusing first on the innate immunity, the differences in glycosylation, I'm not going to talk about Gal-alpha-1,3 Gal, because it's going to be a major subject throughout the symposium. As you know, nearly one percent of serum antibodies are directed against this carbohydrate antigen, because it's not found in man and old world apes. It's a major cause of hyperimmune rejection in xenotransplantation.

Less well talked about is another carbohydrate that is present in pigs, but not present in man, it's a sialic acid which many of you know the term is a terminal sugar on many carbohydrates, but it has different forms. And N-glycolyl-neuraminic acid is present in pigs, but not in man, and has an extra hydroxyl group on the acetate moiety of the common sialic acid found in man, and so its importance as an immunogen is not fully known, but probably needs to be considered as these alpha-Gal knockout pigs are studied.

The other other type of interaction that I've already alluded to are the roles of sugars in cell communication. And there are two major paradigms, one in which a carbohydrate binding protein is present on one cell, and recognizes a unique carbohydrate expressed on another cell, and thereby mediates a cell-to-cell interaction.

And the other type is quite different, in that the carbohydrate binding protein recognizes the carbohydrate on the same cell, and that that -- that recognition can modulate the activity of this protein, and this would be the receptor modulation paradigm.

So to give an example of each, the C type lectin family is the largest carbohydrate binding family in man. There are over 70 members now. The most important group with respect to leukocyte trafficking are the selectins that have already been mentioned. This is a three-member family, the L selectin being expressed on neutrophils and lymphocytes and platelet -- sorry, neutrophils and lymphocytes. E and P-selectin being expressed on endothelial cells, and as we've just heard, P-selectin being named because it's expressed exclusively on platelets.

Well, all three of these selectins recognize a similar carbohydrate called sialyl Lewis-x, and it's shown in the chemical structure here, and the biochemical formula here. There are two key sugars involved in recognition, a sialic acid, an acetylneurominic acid for the abbreviation, and then fucose over here. And both of these are essential for recognition. But there are subtle differences in the specificity of the three selectins that add to the biological complexity of the trafficking interactions that they mediate. E-selectin recognizes just that -- that structure that I showed on the previous slide, Sialyl Lewis-x doesn't seem to matter what it's bound to. P-selectin, however, is extraordinarily specific. In addition to recognizing this structure, it must also have a sulfated tyrosine on the polypeptide to which this carbohydrate group is attached.

L-selectin, on the other hand, also requires a sulfate, but not on a peptide. It requires a sulfate on the sugar. So because of these subtle differences, the expression of these three different types of carbohydrate ligands add to the biological complexity of the trafficking actions that these selectins mediate.

So E-Selectin, as I mentioned, is expressed on endothelial cells and traffics neutrophils to inflammatory sites, and lymphocytes to inflammatory sites.

P-Selectin is expressed on platelets and endothelial cells. It traffics leukocytes to inflammatory sites, and also mediates platelet to neutrophil interactions.

And L-Selectin, finally, is expressed on these neutrophils and lymphocytes, neutrophil trafficking and lymphocyte trafficking to lymph nodes.

I'm going to talk just a little bit more about the inflammatory reaction and lymphocyte trafficking to lymph nodes as examples of these sugars' functions. So as many of you know, in the inflammatory cascade, neutrophils and other white blood cells are pulled out of the vasculature on the activated endothelium lining the blood vessel wall, and the first thing that happens is that the cells roll along the~-- along the endothelium until they are grabbed by other adhesion molecules. They firmly attach, and then migrate into the surrounding tissue, where they can cause the damage, or meet pathogens, as the case may be.

The initial event, the rolling event, is well known to be mediated by the selectins. And if you haven't seen one of these videos, this is a video of a cremaster muscle, where the vessel -- the capillary vessel has been, or venule, rather, has been inflamed, and you can see these white balls migrating along. These are predominantly neutrophils that are being -- which carry the sugar, and are being recognized by the selectin expressed on the endothelial cells lining the surface, and this is the first step in the recruitment of those cells into the tissue.

The other mode that I wanted to show you, just to give you an idea that this same class can mediate very different trafficking, almost like a zip code for lymphocytes to lymph nodes, where the interaction is L-Selectin on the lymphocyte, and the sugar is now on the endothelial cell; where it was the reverse on the previous slide. And the way that John Lyle's group in Michigan has looked at this is by creating a knockout mouse that is being the fucosyltransferase. Transferase is required to add the key fucose residue

on this structure, which is in symbol form is Sialyl Lewis-x. And so by creating those mice, he can look to see what the physiology is, and I just show this picture because it's so dramatic. Basically lymphocytes cannot migrate into the lymph nodes. These are lymph nodes dissected from wild type mice. You can see by contrast that the fucosyl transferase deficient mice can't make the ligand for L-Selectin are very small, they have hardly any lymphocytes, because the lymphocytes can't migrate into the lymph node.

So from a pharmaceutical perspective, you can modulate the activity of the selectins, and then impact their function by either inhibiting directly the Selectin with an inhibitor that mimics the carbohydrate ligand that is recognized, and is a compound in phase three clinical trials by American Home Products. The other idea that is being pursued, there are no compounds yet being developed, but is to inhibit the enzyme, the glycosyltransferase that attaches the key sugar residue, just as in the knockout mouse, so the carbohydrate structure is not present, and the lymphocytes don't traffic.

The other example that I wanted to give is with the other family that I highlighted from the beginning relating to the functions of carbohydrates in receptor modulation. This comes from the siglec family, which is relatively new, largely the -- most of the members in the siglec family have only been known in the last four or five years. And the name comes from -- This, it's an acronym for sialic acid specific immunoglobulin super family-like lectins. So these are members of the immunoglobulin super family. It's a sub-family. They all have in common a terminal domain, which is V set domain, different disulfide bonding pattern from all of the others, and this domain is characteristic of -- or, rather, affords the ability of these proteins to bind sugars, specifically sialic acid and acetylneurominic acid here.

The other thing that is unique about the family is that they have, most of them, I-TM motifs, involved in receptor -- modulation of receptor signaling as inhibitory motifs. So -- there are also differentially expressed, most of them in the immune system, so all but one are expressed in immune cells, and I'm not going to dwell on any of the listings -- the variations here, but you can see they are differentially expressed, so they are believed to play important roles in immune function.

Well, we know, although the functions of most of them are not known, the functions of one of them are, have several important -- I'm going to skip over this slide, and that is CD22.

CD22 is a known accessory protein of the C -- of the B-cell receptor, the most important player being the IgM expressed on the surface of the B-cell. And as you know, that during antigen activation, phosphorylases are recruited which phosphorylate the accessory proteins, and phosphorylation gives rise to strong signal to the nucleus, which allows the cell to be activated, proliferate and start producing antibody.

CD22 recruits phosphatases, and they're -- by dephosphorylation of the complex reduces signal strength and dampens the immune response. Well, what we have been interested in is what the carbohydrate binding domain out here does. How does it modulate CD22's function? And we know that CD22 recognizes a carbohydrate structure where sialic acid is linked to the sixth position of galactose, as shown here in the biochemical nomenclature. This structure is abundantly expressed on both B-cells and T-cells, CD22 being strictly a B-cell glycoprotein -- receptor, rather, in that the carbohydrate structure is synthesized by a single enzyme, abbreviated here, a sialyl transferase that transfers sialic acid to the sixth position of galactose.

Well, by creating a knockout mouse, just as I showed with the Sialyl Lewis-x story, you can ask the question does that carbohydrate structure play an important role in B-cell function? And Jamey Marth and our group collaborated several years ago to create such a mouse. And the mouse is completely normal in most respects, except it has a profound B-cell phenotype. And that is when you immunize with either a T dependent or T independent antigen, you see that wild type mice produce all three isotypes of

antibody quite well over a 30-day period, but the wild type mice have strong immunosuppression regardless of isotype. The group at Novartis, Joan Yorgensen and Andreas Katapotis (phonetic), took a look at a transplantation model, allotransplantation of carotid arteries, and they first stabilized the -- or sensitized, rather, the recipient mice, the cell transferase knockout that don't contain the CD22 ligand, and then the heterozygous recipients, which are normal phenotype by injecting C3H donor spleen cells into the block six recipients. And then the arteries were then transplanted into the sensitized recipients in neointima formation, and antidonor antibody was assessed 30 days later.

As you can see here, the -- the amount -- there is lots of allo antibody produced in the wild type, I'm sorry, yes, in the wild type mice, which were positive for the cell transferase. But in the cell transferase knockout mouse, there is very little allo antibody produced, and as a result, there was dramatically less intimal occlusion in the -- This is -- a representative slide for the -- for the transplant in the wild type mice where new intimal formation from the intimal lining here with the pink with the smooth muscle cell proliferation inside the lumen of the vessel, you can see occlusion of the vessel, 36 percent to approximately 20 percent for the null mice. This is highly statistically significant.

So the idea here, and the reason for doing the experiment, is that if you -- if you, instead of having a knockout in a human, you have an inhibitor to the enzyme, that you could create a similar suppression of immune response.

Well, how is this working? How is the B-cell function being modulated? There are two possibilities for carbohydrate binding protein that I mentioned on the first slide, one is if it's unmasked, it can participate in binding another cell. But on the other hand, if it's masked, it is unable to participate in binding another cell. So which modality is important in this case? And you can distinguish between the two by using a carbohydrate probe. In this case it's a polyacrylamide polymer with the ligand attached, and in the case of a murine model, this special form of sialic acid that I mentioned at the very beginning that is found in pigs, but not in man, and glycolyl-neuraminic acid is required for recognition by murine CD22. We synthesized this and attached it to polyacrylamide. And then if as a control, you can sialidase treat the cells to unblock, or to remove sialic acids from the surface of the cell, and then if there is any -- if any -- any -- if any of the carbohydrate binding protein that was once masked is now free to bind this probe, so you can tell if it's masked or unmasked. When you look at wild type cells, and these, we are only looking at B-cells now, you look at binding of a probe before sialidase treatment. After sialidase treatment there is very good binding of the probe, which means that on wild type cells the -- the CD22 is masked by binding to the ligands, or glycoproteins on the same cell.

In the case of the mice that are missing the carbohydrate ligand, you see that the before sialidase treatment removing sialic acid, there is no -- there is -- I'm sorry, there is binding displaced up from the background, and in after sialidase treatment, there is the same level of binding, meaning that the CD22 is unmasked. So what we think is happening, the current hypothesis, is that the function of the carbohydrate glycoprotein, the glycoprotein ligand on the B-cell is to sequester CD22 away from the B-cell receptor complex, and then thereby modulate receptor signaling, or inhibition of receptor signaling, and in the absence of ligand, CD22 spontaneously associates with the receptor complex and maximally suppresses CD22 function. So in the case of an inhibitor of glycosylation, that you would favor the formation of this complex to suppress B-cell function.

So just as our final slide of comment, ah, I want to leave you with the idea that carbohydrate binding proteins and/or their ligands mediate important leukocyte functions, and the controlling immune function, of course, is an important prerequisite for successful xenotransplantation, and progress and understanding the roles of sugars in biology are likely to -- leukocyte biology are likely to lead to pharmaceutical approaches for controlling immune function.

Thank you for your attention. There are a couple of collaborators that I would like to mention again: Novartis, Joan Yorgensen, Andreas Katapotis, Jamey Marth at the University of San Diego, Lars Nitchke, whose work I just briefly touched on in Germany, and Nicolai Bovin, who helped with the synthesis of the carbohydrate structures in Moscow. Thank you.

DR. SYKES: Thank you very much for bringing us up-to-date on this very interesting and important area that we all need to think more about with respect to xenotransplantation. We have a few minutes for points of clarification. Dr. Salomon.

DR. SALOMON: Jim, one of the things that struck me was you didn't mention the regulation of these various enzymes. So you have this whole series of glycosyltransferases, and then there is another series of sulfation-mediating enzymes as well, right? I mean that, to me, one of the amazing things here is all of a sudden a sulfated group? That definitely didn't get taught in basic Immunology 101, so that the question here is what do we know now about the regulation? I mean, so at the time we do the xenotransplant there is going to be a certain spectrum of these glycolipids and sulfated moieties. How does that change? Do cytokines up-regulate these enzymes? Does that change? Is the landscape going to change as a function of the xenotransplant? Or is it going to be a fixed target?

DR. PAULSON: There are two levels of regulation, one is sort of the set level, so resting, resting leukocytes express one set of carbohydrate signatures that are different from other cells. And when you cross species, you -- we already know about several structures that are present on pig cells that aren't present on man, so that is one level of regulation that needs to be concerned with in this -- This field is already very concerned about that.

The other level is that once a cell is activated, glycosylation changes occur. And that is clearly the case in the immune system. If you look at a cross-section of a thymus, classical staining is with PNA, which is a carbohydrate binding protein plant lectin that recognizes a non-sialated carbohydrate structure. And so cells go from being PNA positive, to PNA negative in the thymus lymphocytes. When the cells go out into the circulation, so that is doing differentiation. When cells go out into the circulation and become activated, they become PNA positive again. And then they become PNA negative again. T lymphocytes, when they go out of the periphery, do not express Sialyl Lewis-x, but when they are activated, they all express Sialyl Lewis-x. And in CD4 Th1 cells continue to express Sialyl Lewis-x, whereas Th2 cells down-regulate Sialyl Lewis-x. So there is lots of regulation -- great question -- lots of regulation of glycosylation occurs afterwards. I don't think there is anything known, really, how much that regulation differs from species to species. And since the immune system is primarily the host immune system, probably the most important regulation concerns the human host.

DR. SYKES: Dr. Scheckler.

DR. SCHECKLER: Let me go back to your opening comments and ask maybe a way too simple question. In the Gal sugar that we heard about at other meetings, that had to be dealt with and sort of taken away from the pig for a xeno of a pig to human to be even considered. I thought I heard you say at the beginning that there may very well be other sugars that the pig has that are automatic immune response, or intrinsic immune response would react to, and we haven't studied the other ones yet, and perhaps we have to have a knockout, knockout, knockout pig in order to get something to work. Is that right?

DR. PAULSON: Well, I didn't want to raise that specter, actually. But yes, I think it's something that needs to be considered, rather than just be surprised about it down the road. So N-Glycolyl-Neuraminic acid is quite famous in the field for -- Man is the only mammal that doesn't express that sugar. One of our esteemed colleagues in the field has almost devoted his life to it. I mean he is doing many other things,

and people don't really pay attention to this aspect of what he is doing. But he thinks that that is the difference between apes and man, is this one sugar difference. And he gets ribbed about it all the time.

But the fact -- I mean the -- The pharmaceutical industry, in particular, companies producing glycoproteins in Chinese hamster ovary cells have been concerned about this, because a small percentage of the sialic acid produced by Chinese hamster ovary cells were on Epo and other glycoprotein drugs, have this sugar. So far they haven't seen any dramatic immune response that would cause a safety problem. But that is a -- a minor percentage in what we're talking about is something that is very controlled, it's a minor percentage of the carbohydrates on that glycoprotein. The worst thing that would happen would be they would be removed, so be going expressed on the surface of an organ maybe is a different thing. It may not be a problem at all, but it's just a -- a -- and there may be people in this room who know more about it, and already considered it, in fact.

DR. SALOMON: I just wanted to point out what is fascinating here is this is something that wouldn't point out pop out of the baboon or cyno or rhesus studies. This is only something that you'd only have to deal with when we go up to the clinical.

DR. PAULSON: That's a very good point.

DR. SYKES: Do you know if any of the 10 to 20 percent of human natural antibodies that are not against Gal, and are against pig, are against this determinant? Has anybody looked at that, or induced antibodies in people who have been exposed to xenogeneic tissue?

DR. PAULSON: I don't believe there are naturally occurring antibodies, because I think the reason that we all have very high titers of anti-Gal antibodies is because the structure occurs in bacteria, and we are exposed to it. But I don't believe that there are naturally occurring antibodies. I guess what we would be concerned about is that they be elicited upon the transplantation.

DR. SYKES: Well, thank you very much. One more quick question. Dr. Chapman.

DR. CHAPMAN: **F**ollowing up on Dr. Scheckler's question, my assumption at the beginning, I mean basically what you're talking about here is approach to the first prong Dr. Robson was talking about in particular, which is identifying the things on the surface of the tissue you put into someone that kick off these problems, and trying to change them in some way. And the classic way has been using transgenic techniques to take things away, or add them in to make that surface more compatible. But did I properly understand your concluding remarks, which implied to me that you thought actually these understandings might be as effectively or more effectively used to develop drug therapy? Sort of the third approach Dr. Robson talked about? Where you might be able to develop drugs that you use the way we use coumadin in people who have artificial heart valves or atrial fibrillation to smooth things out and prevent these problems.

DR. PAULSON: Yeah, did you understand me. You did understand me properly, I think. The reason I raised that subject was in the context of ways to regulate the immune system, that by approaching mechanisms the sugars are involved with is another way to develop immunosuppressive drugs.

DR. CHAPMAN: And where is that idea in the spectrum of development? Is it still a hypothesis? Is it in the basic research stage? Or are there actually candidate possible drugs that are being identified?

DR. PAULSON: Well, as I mentioned, there is one phase three trial that is really a parenteral inhibitor for acute and -- or acute indications by American Home Products and Genetics Institute with respect to development of drugs for xenotransplantation. It is very early in its basic research stage, so it's not on the

horizon, not on the immediate horizon.

DR. SYKES: Okay, well, thank you very much. There will be time for more questions later.

(break)

DR. SYKES: We'll now move on to the next speaker, Dr. Jeffrey Platt. Dr. Dalmasso's and Robson's talks have both touched on how the coagulation and complement pathways interface with xenogeneic endothelial surface, and Dr. Platt is going to tell us more about what goes on at the level of the endothelial cell. Dr. Platt?

Agenda Item: Endothelial Cell-Host Interactions in Xenotransplantation

DR. PLATT: Dr. Sykes, Dr. Vanderpool, ladies and gentlemen, it's a pleasure to be here. Perhaps we can turn the lights down a bit, and hopefully this is in focus.

If this symposium had occurred 100 years ago and if it were possible to show a slide, which of course then it was not, it might very well have begun with this slide that shows three gentlemen who were veterans of the war between the states. Now, there are a number of notable aspects to this photograph, perhaps the hats or the facial hair, but of course you might, like I, have most been struck by the fact that the gentleman in the middle is missing his right leg. This was the most serious salient problem in medicine and surgery at the turn of the 20th century, and that was that if a blood vessel became injured due to a traumatic wound, that the extremity bearing that blood vessel would have to be removed because otherwise gangrene would ensue, and the injured individual would ultimately die. And so when we see ancient photographs of veterans of wars, we commonly do see individuals who have had amputations of their extremities.

Now, this problem was solved in the early years of the 20th century by a technical advance which we refer to as the vascular anastomosis. I apologize that this slide is backwards, but the vascular anastomosis really refers to the sewing together of the cut ends of blood vessels. This was an enormous challenge 100 years ago, but something of course that we take for granted today. Those who developed the vascular anastomosis though realized immediately that this was not simply a technical advance that would allow the prevention of removal of extremities and so forth. They realized this was the critical procedure that would be needed to allow the transplanting of an organ from one individual to another. And since this advance was undertaken and since transplants were undertaken a few years hence, the problem of blood vessel disease has really been at the forefront of the entire field of transplantation, not only xenotransplantation.

Now, this shows you the cut end of a blood vessel, and it helps me to illustrate for you why this problem is so important. When a transplant takes place of an organ from one individual to another, the blood of the recipient courses through the transplant and is exposed immediately to the lining of blood vessels. This lining is called the endothelium, and the cells that comprise this lining are called endothelial cells. And so these are the first cells in the graft to confront the immune and inflammatory components of the recipient, and we now understand that this is the most vulnerable point in a transplant, whether it is a xenotransplant or an allotransplant, a human-to-human transplant or a mouse-to-mouse transplant.

When immune and inflammatory elements are brought to bear on the endothelium of a transplant, various problems can ensue, which you have already heard about from previous speakers, such problems as hyperacute rejection and acute vascular rejection and chronic rejection. These types of rejections are diseases of blood vessels, and we think, to at least a certain extent, these problems reflect abnormal behavior of the endothelial lining of blood vessels, and it has been our thought now for over ten years, as

taken from this front cover of a journal in which we published a paper many years ago, that if we could understand at a molecular level or a cellular level the events that were ensuing on the endothelial lining of blood vessels, that perhaps we could devise strategies that would specifically allow successful transplantation, in this case of xenografts, but perhaps more broadly, for other medical problems that could not otherwise be undertaken.

Now, what happens to the endothelium of a xenogeneic blood vessel following transplantation of a xenogeneic organ has already been described for you by previous speakers, and I won't take your time to reveal it again, but I do want to illustrate in this slide some of what can transpire. This is the inside of a blood vessel just like the previous blood vessel, but this is from a xenograft, and these are endothelial cells, and here you can see large spherical things that are sticking to these endothelial cells. These are platelets, and these platelets have been incited to interact with the transplant. So you can see that the blood vessel is extraordinarily abnormal following a xenograft, whereas a normal blood vessel you would just see a clean, unobstructed endothelium leading into the blood vessel. The other process, which was so eloquently described by Dr. Robson, is that in addition to the interaction of platelets with the endothelial cells, there also occurs obstruction of blood vessels by fibrin clots, and this is an immunopathology photomicrograph showing you fibrin that is lighting up in apple green, and you can see that this fibrin has literally plugged up the blood vessels of the graft. This is a very typical picture of what one might see in a xenograft, and it is pictures such as this one that suggested to us so many years ago that perhaps the problem of xenotransplantation could to a certain extent be reduced to the problem of endothelium, and an understanding of how these kinds of clots arise could contribute to a solution to the problem of xenotransplantation.

Now, endothelium, or perhaps more broadly, blood vessels, are not simply passive tubes in which blood passes through the various organs. We now know that endothelium actively participates in preventing coagulation, preventing inflammation and preventing immunity. And if you look here on the left where I have labeled this Resting Endothelium, you can see some of the molecules which you have already heard about and which we think contribute to this resting state that allows blood to remain fluid and blood vessels to remain open, molecules such as thrombomodulin and prostaglandin I and CD39 and so forth.

It has been known now for about 15 years that this is not the only condition in which endothelium exists. Endothelium can also be stimulated by noxious substances or by molecules that we make in our body to become activated, and when endothelium is activated, its behavior changes dramatically, and for the large part it is the opposite of what I just described for resting endothelium. Activated endothelium, for a variety of molecular reasons, stimulates coagulation, promotes and encourages inflammation, and allows immunity to ensue. We thought perhaps 12 years ago now that the problem of xenotransplantation and all of the things that you've heard discussed might arise because the endothelium of a xenograft is activated. Now we know this is rather an oversimplification. In fact, the activation of endothelium is something that occurs every day in all of us. For instance, those of you who have hay fever probably experience activation of your endothelium in your nose, and this type of endothelial activation some people have linked in the literature to hyperacute rejection. Now, those of you who have hay fever are not about to reject your nose. Maybe you wish you could reject your nose, but the fact is that it is an oversimplification to think that the simple one-way pathway between simulation of a blood vessel and activation leads inexorably to rejection. Clearly there are other aspects that are important, if not essential, for the rejection pathway, but this model, this concept, has allowed us to study the various events that go wrong in a xenograft at the cellular and molecular level and at least have allowed us to test some strategies for interfering with the various pathways that we think might occur.

Now, perhaps the most important point that I want to make during my brief time with you this morning is on this slide and the next slide. In a sense it is counter to what you have heard this morning before me and perhaps what you will hear later. It is easy, well, if not easy, it has simply been accomplished that

we can identify nearly an infinite number of things in the blood that can interact with and damage a blood vessel and stimulate endothelium to become activated. Now, on this slide I list some things that you've already heard of. You've heard of platelets which can interact with endothelial cells and stimulate those cells. C5a, a cleavage product of the fifth component of complement, which Dr. Dalmasso has told you, is generated in transplants and xenotransplants, antibodies attaching to carbohydrates, whether it's Gal alpha 1,3 Gal or other carbohydrates, macrophages, other white blood cells interacting with the cell surface. Virtually every element of the blood can potentially interact with a blood vessel of a xenograft, but the complexity doesn't end there.

Let's just consider for a moment a few of the pathways that are excited by these various elements of the blood. Here I show you antibodies interacting with structures that we described some years ago are integrin-like structures in the endothelial membrane. This activates several signaling pathways. Here is C5a upper in the left-hand corner and the C5a receptor, or G protein coupled receptor, shown here, and here are the various pathways that are activated. Here is the membrane attack complex of complement. This is enormously complex, and for the sake of this slide we have reduced this to only a few of the many interactions that could potentially occur between the blood of the recipient and the blood vessels of the graft. And one could focus on one pathway and say, well, if we identify this molecule, PI3 kinase, perhaps if we develop an inhibitor for this, we'll solve the problem. Well, obviously that is folly because there are an infinite number of ways in which blood cells and components of the inflammatory system can interact with blood vessels.

So the message that I first want to offer you is this one. There are two ways of looking at the immune and inflammatory interactions with endothelium, and one is modeled on this slide, and that is the possibility that there are an infinite number of interactions and incompatibilities. Every protein in the human is incompatible with the pig. Every sugar is potentially unique. And so there may be an enormous, if not infinite, number of ways in which the recipient's immune and inflammatory elements act on the xenograft. And if this is the case, if one has to devise a therapeutic strategy for dealing with each one of these, these strategies are going to be manifold, if not infinite. You're going to need to block this pathway, this pathway, this pathway, and so on, and so forth. Now, this makes a great joy for those of us who are scientists because we can focus on an infinite number of pathways, but it's rather a pain in the neck for those who want to make xenotransplantation succeed.

But there's another possibility that I want to suggest, and that is that the pathways that can be defined as being abnormal are not necessarily all going to cause injury and demise of a xenograft, and that perhaps one can reduce the problem to a series of seminal events which are depicted here. I'm afraid this pointer isn't working. I don't know if anyone has another one that I can use. Thank you, Bob. The possibility that one can reduce the problem to a few seminal events, which then give rise to the extraordinary complexity, and if that is the case, then one might even imagine interfering with the one seminal event. So to me an important question in the field of organ xenotransplantation is to ask, are there a few, one, or a limited number of seminal events that cause the demise of the graft or do we need to deal with the extraordinary infinite complexity that I suggested on previous slides? So we've looked for this reductionist idea and whether it might possibly be correct.

Now, this slide is a very old slide. It is taken from work that was carried out over a decade ago. And what it depicts is coagulation on cultured endothelial cells, and it's asking the question, if you take human plasma, the liquid part of human blood, and you place it on pig endothelial cells, does it clot the same as human plasma would clot? Well, here's the human plasma. You can see it takes, I don't know, 140 seconds for clotting to occur, and if you just take -- I'm sorry, here is pig plasma. And if you just take human plasma and put it on the pig cells, clotting occurs in less than 50 seconds. So clotting occurs much more rapidly with human plasma on pig cells than pig plasma, and this ultimately led us to find that tissue factor is produced by the cells. Now, when we only used human antibodies or we only allowed the

complement system to interact, coagulation occurred at the same rate as with pig plasma. So this suggested the possibility that there might, at least as far as the plasma is concerned, be a seminal event -- as we now understand it, the activation of complement -- that would drive this hastening of coagulation.

Here's another example. This just shows after complement is activated on cells, it gives rise to various events which were depicted by previous speakers. I won't explain this, but what this slide shows you is that there is a single protein that is made by endothelial cells in this system that gives rise to a myriad of changes in the endothelial cells, and if we either inhibit complement or we inhibit that one protein, we can prevent the nearly infinite number of changes that one could describe in endothelial cells. Now, this is only cells in culture. These are scientific experiments that are interesting to us, but the point I want to make is that there exists at least the possibility that one can identify a few seminal events to the extent that this system represents what would occur in a xenotransplant.

Now, we've also done experiments in whole animal systems to test the same thing, and I just want to show you here is a way that we detect changes, abnormalities in blood vessels, and that is that the red blood cells in the blood change their shape when blood vessels become damaged. And here on the left you can see normal red blood cells, and here on the right, I don't know if you can see this in the back, but the red blood cells are fragmented, and their shape is getting fairly ugly. And here we looked at xenotransplants where we made a limited number of modifications of the recipient and the donor, and we asked whether there's a difference in the shape of the red blood cells. And here you can see the red blood cells are completely abnormal, in large numbers, until this point when the graft rejects, and here when rejection is prevented again by a limited number of modifications, the abnormality in the blood vessel is not seen. So this suggests, at least indirectly from in vivo studies, that it may be possible to undertake a limited number of changes either in the donor of the transplant or in the recipient that will prevent the demise of a xenograft and that we can use the endothelial events possibly as a signal for these.

Now, here's another experiment in which we've interfered with just one signal event that we think, and instead of coagulation, this is a normal pattern of blood vessels, and here is an abnormal pattern, and instead of this abnormal pattern, we've gotten a normal pattern because we've interfered with just one pathway which we think is seminal in xenografts.

So the point that I want to make to you is that it seems to me to be essential to understand are there an infinite number of problems that we need to solve, because that will probably take an infinite period of time, or are there a few problems which give rise to the great many problems that a xenograft has? And if this is this case, this is a solvable circumstance. And I think, my own opinion is, the only way to determine which of these is true is to carry out the experiments in xenografts and not to rely only on experiments that we carry out in test tubes.

Now, I want to mention another subject, which is I don't want to suggest that if there are an infinite number of problems, there is no solution to it. There may be a solution. I want to discuss for a few minutes the transplanting of organs across ABO blood group barriers. You know, the A and B blood groups that we have -- perhaps some of you are blood group A, and some of you are blood group B -- we all have antibodies in our blood against the blood groups that we don't have on our red blood cells. So I happen to be blood group O, and therefore, I have antibodies against blood group A and B. And it's been known for a great many years now, or at least it's been thought, that if you were to transplant an organ from one person to another, and the organ had a blood group in it, and the recipient was someone like me with antibodies against that blood group, that the outcome would be terrible. And here on the left you can see an organ where there is painted on the endothelium blood group A. It happens that the level of expression of the blood group antigens is about the same as the level of expression of this Gal alpha 1,3 Gal, and the concentration of antibodies in our blood against the blood groups is about the same as the concentration of anti-Gal alpha 1,3 Gal antibodies. So in a sense the transplanting of organs across blood

groups is a model for what might occur, at least as far as antibody antigen is concerned, in a xenograft.

Well, as I said, it had been thought for many years that you couldn't carry out such transplants, and yet in the mid 1980s a number of groups started to conduct these transplants by temporarily removing anti-blood group A and B antibodies from the circulation, and what was found was remarkable, that when these transplants were carried out, the antibodies came back to the circulation, and yet the transplants continued to function. The vascular disease that I depicted to you earlier, which happens to be in a xenograft, but could have been in an allograft, didn't occur. So Mike Chopek and I in the mid 1980s mused about this because there were a few patients at the center where we were located that had gotten these ABO incompatible transplants, and we carried out some primitive experiments, here showing that the blood group antigen is retained in the graft. So it wasn't just that the antigen was no longer expressing. We could show, in answer to a question that was raised by another gentleman earlier, that the antibodies were roughly the same. If anything, the antibodies could even come back at a higher level in some cases than had existed before the transplant. We ultimately called this accommodation and mused about what possibly could underlie accommodation. But the idea of accommodation is what's really important, and that is that even if there are an infinite number of things which act to injure a xenograft, it is at least possible based on this that a xenograft could undergo accommodation and thus resist these various myriad insults possibly.

Now, I won't take your time this morning. We could devote the entire lecture, in fact, we could devote the entire morning, to discussing what possibly underlies accommodation, but I want to suggest to you that there are a number of us who are thinking about this and asking what is the molecular mechanism? Because think about it. If an organ could resist rejection across an ABO blood group barrier or a xenograft -- and it has been achieved in some xenografts, as Gus Dalmasso mentioned -- then perhaps we could make organs resist other kinds of vascular disease. So we have much interest in understanding what accommodation is, and we are developing models that could explain at a molecular level what these processes might be that allow this resistance or accommodation to occur, and I just list a few of these on this slide.

Now, in closing I want to offer you one further comment regarding blood vessels and xenotransplants, and that is the problems that I have been talking about and that the speakers before me spoke about are problems of vascularized organs, but the critical question in understanding the biological response to transplantation is to know what kinds of xenografts we're talking about, dispersed cells, such as hepatocytes, free tissues, such as pancreatic islets, or whole organs, that although we think that all xenografts excite the same type of immune response, all xenografts are not equally susceptible to all of the elements of the immune response. And the things that I have talked about and the previous speaker are unique vulnerabilities of organ xenografts, and as far as we know, they are not shared by xenografts that consist of cells or isolated tissues except perhaps under certain limited circumstances. The reason for this is depicted on this slide. In an organ transplant where you have sewed together the recipient's blood vessel and the donor's blood vessel, all of the elements of the recipient can, as I've shown you, act on the blood vessel. In a cell transplant the blood vessel comes from the recipient. It grows into the graft from the recipient. So all of the molecules on the blood vessel are those that are already in the person or animal's body already.

Here I show you a slide I took from a paper published a number of years ago by Gunsles, et al., who were interested in studying hepatocyte liver cell transplants for treating a disease in which the blood cholesterol goes way high, and those who have this disease, which is called homozygous hypercholesterolemia, die a very early death, in their teens or early 20s, from atherosclerotic heart disease presumably due to this cholesterol. And right now if someone has this disease -- and fortunately it's rare -- they get a liver transplant. So you transplant the whole liver, the largest organ in the body. You transplant just to correct a genetic defect in a single molecule in the patient's liver. Well, the idea was perhaps you could deal with

this problem by xenotransplantation, and here they've transplanted hepatocytes from the pig into rabbits that have that, and here you see the outcome. This shows the blood cholesterol level in these rabbits, and it's very high in this group of rabbits and much lower in this group, and the only difference is that the group with the lowered cholesterol got transplants plus cyclosporine, which is an immunosuppressive drug, whereas this group of animals didn't get cyclosporine, but did get hepatocytes. And I show you this to point out here is an example of a xenotransplant that is alive and functioning with less immunosuppression than you would give a human who got a kidney transplant or a heart transplant.

Here's another example, even more dramatic. These are xenogeneic hepatocytes in one of our own experiments. Here's a blood vessel. This animal isn't even getting immunosuppressant. There is no modification in this case, and this animal has liver failure. And without these hepatocytes the animal would be dead. And if you look in the blood, you can find very high levels of antibodies against the pig, and yet it doesn't seem to disturb the survival or the function of the cells.

So when we think about the problems that we've heard about this morning, these are problems that, for the most part, are restricted to organ xenotransplants or to certain cells, such as bone marrow cells, as Dr. Robson described, that are put directly into the blood, but if you can get cells into the body outside of blood vessels, then it is possible to avoid these various types of problems, and one confronts then cellular rejection as the major impediment to xenotransplantation and one that we may or may not be able to deal with with the same immunosuppressant drugs that are currently in use. Well, I thank you for your attention, and I'd be glad to answer whatever questions you may have.

DR. SYKES: Thank you very much, Dr. Platt, for, as always, a very elegant presentation and for integrating much of the information that we've already heard this morning into a larger picture. Does anybody have any points of clarification? I would ask you to really stick to this kind of question at this point because we are running behind time, and we'll have lots of time during the panel discussion to ask more detailed questions. Okay. I think then in that case we'll move on to the next speaker, who is Dr. Robert Zhong from the University of Western Ontario. He's going to update us on the current status of pig-to-nonhuman primate transplants with a focus on the role of immunosuppressant drugs in large animal xenotransplantation. Dr. Zhong?

Agenda Item: Current Status of Pig-to-Nonhuman Primate Transplants

DR. ZHONG: Dr. Sykes, Dr. Vanderpool, in the next 25 minutes I would like to give a brief overview of the current status of pig-to-nonhuman primate transplants. Okay. If the pig has a choice, he would like to be a human donor instead of ham, sausage or pepperoni. Before we start a clinical trial, we would like to show the public xenotransplantation is effective procedure for the patients with end stage of diseases, therefore, it is important to show the pig organ, such as the kidney, is able to support a nonhuman primate, such as a baboon, for a long period with clinically acceptable immunosuppressive protocol. Our group has been working in this field for many years. I would like to show you some progress. We transplanted a human DAF pig kidney into a baboon. In the recipient we removed the baboon's own native kidneys to create a life-supporting model. At the beginning we used the protocol which was originally developed by the previous Imutran group. We treated the recipient with cyclophosphamide, and we gave the animal cyclosporine, steroids, RAD, a new analogue of rapamycin, as maintenance therapy. With this protocol we were able to achieve up to 40 days survival. The serum creatinine level in the baboon remained normal in the first 20 to 30 days, then gradually elevated. Eventually these animals died from uremia between 30 to 40 days. These microscopic pictures show you the sequential biopsies from a human DAF kidney xenograft in the baboon. You can see that before day 23 this kidney graft has a normal histology. On day 30 the graft develops a typical feature of acute vascular rejection. As you can see here, the wall of this small artery is getting very thick. Interestingly, there are very few infiltrating cells, even at the terminal stage, but the vascular changes is very remarkable at that time. The immuno

stainings show there is a massive deprivation of IgG, IgM in the pig kidney, and these figures show the xeno antibody as measured by antiporcine red cells, hemolytic assay, anti-Gal, IgG, IgM. And you can see the xeno antibody was significantly elevated, which was associated with rejection.

Up till now the protocol, including cyclophosphamide, cyclosporine A, steroids, with or without a fourth agent, has been the standard protocol for most of the transplant centers in the pig-to-nonhuman primates transplant. Despite great efforts that have been made, the best results were a mean of 30 days survival in the pig-to-nonhuman primates kidney or heart model. Once we reached to the 30 days, we just like hit a stone wall. Acute vascular rejection and the drug toxicity remain major barriers. Cyclophosphamide is the major component in this protocol, but unfortunately this drug has many side effects, such as thrombocytopenia and GI toxicity. As showing you in this picture, one of our baboons developed multi-organ bleeding, which we believe is due to the thrombocytopenia caused by cyclophosphamide.

In the recent few years we have developed a totally new technology in this model, first using GAS, a Gal analogue, to absorb anti-Gal antibodies, and we eliminated cyclophosphamide in this protocol, and also we used FK to replace cyclosporine A. The GAS914 is a polymeric form of alpha-Gal trisacharide. To make the story simple, this molecule structure very much like Gal, we found that intravenous infusion of this agent is able to effectively absorb anti-Gal antibody in the baboon, circulating blood. As showing you in this picture, with one week pretreatment of GAS, we can significantly reduce the baboon preform antibody to the baseline. Xeno antibody remained normal as long as GAS was given continuously. The aim of this protocol was to determine whether intravenous infusion of GAS would ameliorate acute vascular rejection in this model. The baseline immunosuppressive protocol included cyclophosphamide, cyclosporine A, RAD and steroids for all the groups. Group 1 received no GAS. Groups 2 and 4 received the GAS with a different dose and a different duration. In the non-GAS-treated group three of four animals died of acute vascular rejection. Average survival in this group was 19 days. In contrast, in the GAS- treated animals, only 25 percent of the animals died from rejection, and also we found the pathologic changes of acute vascular rejection was much less severe compared with those in the non-GAS-treated group, but we were not able to improve the results using the GAS, improve the survival in this group.

These pictures show you the gross findings at necropsy in both groups. The top picture shows you a pig kidney which was transplanted into a baboon, and there was no GAS therapy. On day 40 you can see the kidney was completely necrotic. The bottom picture shows you a pig kidney which was transferred into a baboon with GAS treatment. On day 37 apparently this is a good-looking kidney, and in fact, this baboon died of bleeding due to the biopsy. These microscopic pictures compare histological changes between the GAS and the non-GAS-treated groups. There was not much difference in the early stage. Interestingly, there's a remarkable difference at the terminal stage. The left pictures show you the graft in the non-GAS-treated group. You can see massive hemorrhage, cell infiltration. In contrast, in the kidney graft treated with GAS at the similar days, there was no hemorrhage and also very few infiltrating cells. Then next we eliminated cyclophosphamide with rabbit antithymocyte serum. Our hypothesis was the RATS is more effective and less toxic than cyclophosphamide in this model. This agent is actually very similar to ATG and AOG, which are widely used in clinical transplantation. This drug is the antibodies directed against the multiple epitopes on the human lymphocytes. It has been demonstrated that this agent is very effective to prevent acute vascular rejection following clinical transplantation. The control group used cyclophosphamide as induction therapy, and the study group used RATS instead of cyclophosphamide. The immunosuppressive protocol was very similar among these three groups. In the two animals in the cyclophosphamide group, they all died from rejection at day 28. In contrast, only one animal in the RATS group actually died from rejection. And the rest of them died of other causes which are not associated with rejection. And in fact, many of them died from infection. Retrospectively we believe we over-immunosuppressed these animals, and the survival was no significant difference among these three groups. We found that the pattern of rejection was significantly reduced by using the RATS.

In the cyclophosphamide group all the tissues had severe evidence of acute vascular rejection. In contrast, the tissues collected from the RATS-treated group only had minimal evidence of acute vascular rejection.

Finally we modified this protocol. We did several things. First of all, we significantly reduced the doses and the duration of RATS as well as for GAS, and we replaced high doses of cyclosporine with FK, and we replaced RAD with MMF. And the doses we used in this protocol, like FK and MMF, were very similar with the doses we used in the clinic, and we believe this is a clinically acceptable protocol. And why we used FK is because we all know FK is more potent than cyclosporine A in preventing allo and xenotransplant rejection. Perhaps more importantly, FK is antithrombotic while CsA is prothrombotic.

The first baboon treated with this protocol lived to 75 days, which is far beyond the previous protocol using cyclosporine. This baboon's name is Zach. The picture was taken on day 36. At the time he was very healthy and a baboon with normal renal function. And also we found that the hematology profile in this baboon was completely normal during the 75 days follow-up. You can see the platelet number was completely normal. Same with WBC and hemoglobin. We never see such a normal pattern of hematology in the cyclophosphamide-treated group. These pictures show you the biochemistry profile in this baboon. You can see the sodium, potassium, pH, were completely normal during the 75-day treatment. That indicates the kidney was working in this animal during the entire follow-up period. However, we did find a massive protein loss in the animal urine starting at day 15, as indicated in the red color line. To compensate for the urine protein loss, we had to give human albumin to maintain the normal serum albumin level. We don't know why, but we believe this is due to the low degree of human rejection. Unfortunately, this animal eventually died from acute vascular rejection. There was some deprivation of IgG, IgM, C3 and fibrin in the terminal specimen. And we also found the antibody level was elevated when the animal developed rejection.

I also would like to briefly overview the important contributions from other centers in the past two years. For example, the Nextran group, in collaboration with the Mayo Clinic, developed a Gal conjugate, and the Boston group developed a bovine serum albumin conjugated to the Gal. These two agents are very similar with GAS, which are able to effectively absorb anti-Gal antibodies. And the David Cooper group also reported that anti-CD154 monoclonal antibody, which is against CD40 ligand, is able to effectively prevent inducible IgG. I think this is a very important contribution in this field.

Where we are now. It is clear that hyperacute rejection can be overcome by using transgenic pigs. Prolonged survival, 75 days in the baboon, 90 days in the monkey, can be achieved using a clinically acceptable protocol. The graft function, at least for the kidney and heart, is unlikely a major problem, however, acute vascular rejection remains a major problem.

This slide compares the allo immune response with xeno. The allo immune response is primarily adaptive immunity, such as T cells and inducible antibodies. In contrast, in xenotransplantation we are facing both adaptive immunity as well as innate immunity, which includes natural antibodies, complement, NK cells and macrophages.

In the past few years we have developed several new technologies, such as transgenic pigs, GAS, RATS, et cetera, but we are wondering why we are not able to achieve a reliable long-term survival in the pig-to-baboon model. I would like to show this cartoon. Let's say in this barrel there's ten holes, but a block in one hole certainly cannot prevent leakage of water. But just to point to Dr. Platt's lecture, maybe the one hole is the key hole. If we can block this hole, we may can solve the problem. But so far we don't know which one is the key hole. I have to admit we have problems in our animal model. The drugs are designed for man and not for the monkey and the baboon. For example, for the baboon it requires ten times the cyclosporine level to inhibit the in vitro lymphocyte perforation. The transgenes are human and

less effective to protect xenografts. And our preliminary results showed the baboon developed the human transgene, such as human DAF, around day 30. And we only have limited diagnostic and therapeutic procedures for the animal. For example, the monkeys I'm using for transplant are only three kilograms. This is just like an infant. We do need pediatric intensive care, but unfortunately we even do not have a lot of basic equipment, like x-ray, ultrasound, et cetera. Most of the baboons I'm using for transplant are wild-caught, and we found preform antibody in these animals is twice as high compared to human preform antibody. These baboons are living in the jungle. They have multiple infection with virous bacterial particular parasites, so their immune systems actually are activated. So I don't say we should forget the nonhuman primate model, but my view is the results may be better in the human using the current protocol.

And what's in the future? First of all, we need to further modify the pig. Ideally we need a knockout Gal. I think that Julia will tell us this story in the afternoon. And certainly we need a better immunosuppressive agent targeting B cells. The current antirejection drugs, like cyclosporine and FK, are very good at inhibiting T cells, but they are less effective in inhibiting B cells, so we need some drug either targeting B cells or masking B cells. And of the two agents currently I'm working on, one is LF, which is the analogue of deoxyspergualin. I will elaborate in a minute. And also I agree with the previous speaker. We need to inhibit complement, such as using the monoclonal antibody C5. And finally, we need induction of tolerance.

In the beginning of this year the two groups reported development of alpha-Gal knockout pigs. It will be very interesting to see what happens when we transplant these pigs into the baboon. The mechanism of this agent is likely to inhibit NF-KB translocation, and we have data to indicate this drug is able to prevent DC's maturation as well as to inhibit T cells independent antibody production.

This slide shows the antibody level in the rat-to- mouse model on day 6. You can see in the untreated animal, the animal had a very high IgG and IgM level on day 6. Cyclosporine only partially inhibited IgG. Cyclosporine was not able to inhibit IgM. We know that IgM is T cell independent. And the LF effectively inhibited both IgG and IgM.

This is the drug I gave to my baboon after transplant. Certainly this is not the way we should do for future clinical transplantation. We need to develop a therapy either to create accommodation or tolerance. I will leave this to Dr. Sykes.

In the last century to put a human on the moon and successful development of allotransplant were considered a miracle. In this century if the pig organ can be transplanted into the human to save the human life, this will be considered another miracle in human history. This is the reason why we are here. It sounds like a dream, but I would say it's worth the reality if we can figure out the mechanism of xenotransplantation. I want to show you a monkey. This monkey's name is Oscar. We transplanted a baboon liver into Oscar, and Oscar lived 1,073 days. Certainly he is the longest surviving monkey with a baboon liver. If we have another Oscar with a pig kidney who lives for a year, at that time the xenotransplantation will be a clinical reality. Certainly this is teamwork. I will not read all of the collaborators' names, but I would like to emphasize this project was collaborated with a team at University of Toronto under the direction of Dr. Levy. Dr. Levy is here, and he will give a presentation this afternoon. Thank you for your attention.

DR. SYKES: Well, thank you very much, Dr. Zhong, for updating us on what really are tremendous advances in obtaining xenograft survival in primate models, and also for pointing out what is limiting us now, still this acute vascular rejection problem. And I think your talk reinforces what Dr. Platt emphasized, that if we can target critical areas involved in xenograft rejection, that we should be able to overcome these problems.

Are there any points of clarification that anybody would like to make at this point? If not, I think what we'll do is instead of breaking after my talk, people have been sitting for a long time, and so we're going to take a 15-minute break right now. But please come right back at 11:15 because we really are already behind time, and as you know, we have a rather dense schedule. Thank you.

(Recess)

DR. SYKES: Okay, well, I have 25 minutes in which to cover the immune response to xenografts and induction of tolerance, which is a lot to cover in a relatively short time. Luckily some of the earlier speakers have really paved the way, and Dr. Zhong, I think, has highlighted some of the mechanisms of immune rejection particularly the importance of acute vascular rejection and the need for tolerance, so that helps my job, make it a little bit easier. So I'll go fairly quickly through the parts that you've already heard something about.

Xenograft rejection can occur through a variety of processes, one is hyperacute rejection, which is initiated by natural antibodies, as you've heard, and occurs within minutes to days.

Then acute vascular rejection is the same entity that I'm calling delayed xenograft rejection, is something that occurs later, days or even months later, as you heard today in Dr. Zhong's studies. In most cases, it's antibody dependent, although there are examples where it occurs in the absence of antibody and in addition to antibody, natural killer cells and macrophages, which I'll come back to, seem to play a role.

Finally there is acute cellular rejection, which is mediated by T-cells, and I'll tell you more about that, and chronic rejection, which you've also heard something, about a much later process.

Now, what are the cells and molecules involved in this process? Well, I mentioned antibodies already. Antibodies can occur in a natural form, like the anti-Gal antibodies, or they can be induced by a xenograft; and anti-Gal levels increase and other specificities appear, when a xenograft is placed. Natural killer cells are cells of the innate immune system, which means that they pre-exist without prior exposure to the donor. They have reactivity against it. And as I'll say later, these cells of the innate immune system are very important in xenotransplantation, likewise, macrophages.

Gamma-delta T-cells, a subset of T-cells involved in innate immunity, also probably plays a role in xenograft rejection. The adaptive immune system is that response which comes up after antigen exposure, and that includes the classical T-cell response, which I'll tell you more about, and as I said, induced antibodies.

Now, just to mention the T-cell response, you haven't heard much about it thus far in this entire committee, and the reason for that is that xenografts have all been rejected by either hyperacute or acute vascular rejection. And before -- and even in animals that are strongly immunosuppressed, and so there has been very little opportunity to take a look at T-cell mediated rejection in vivo, because the process never happens, because it's precluded by other rejection mechanisms like AVR. However, there is at least one in vivo example of cellular rejection having occurred in a heavily immunosuppressed primate receiving a porcine graft.

And in vitro a lot of studies have shown that in fact, there is a robust human T-cell response to Porcine xenoantigens, and that overall this response is even stronger than the very strong T-cell response that exists to allo antigens. And this response consists of both direct recognition of porcine antigens that are presented by porcine cells, and what is called the indirect pathway of recognition, wherein human antigen presenting cells pick up porcine antigens and present them to human T-cells. This is a very important

response, more important for the xeno response than for the allo response. And it is this potency of the T-cell response that makes us think that tolerance will in fact be necessary in order to get xenograft acceptance.

Now why is this xenograft response so strong? Well, one reason is that there are more antigenic differences between a xenogeneic donor and an allogeneic donor. That is sort of obvious, and studies have borne that out. In addition, all the interactions that have been looked at between human T-cells and porcine cells as antigen presenting cells, seem to function either perfectly well, or almost as well as the human-human interactions, and I'm not going to go through all of these, but these include T-cell receptor, co-receptors and co-stimulatory and adhesion molecules. All of these, when looked at with porcine ligands, work very efficiently, unfortunately for us.

So how can we overcome T-cell mediated rejection? Well, one would involve chronic immunosuppressive therapy, but as I said earlier, the T-cell response to xenografts is stronger than that to allografts, and I think there are examples where this even applies to nonvascularized grafts. But certainly it is true for vascularized xenografts. And we believe that excessive nonspecific immunosuppression would be required to prevent this response, if we ever got a graft out far enough to see the response.

And so we think T-cell tolerance is going to prove to be very important. Tolerance avoids chronic immunosuppressive therapy, which if given in excessive amounts would lead to toxicity, such as nonspecific immunosuppression, predisposing to infections and cancers. So -- Oops, wrong way.

So how does one induce T-cell tolerance? Well, there are two major ways one can think of T-cell tolerance. One is called central tolerance, and what that means is tolerizing T-cells during their development when they're at the immature stage of development. And there are two ways of being looked at to induce this. One is induction of mixed hematopoietic chimerism, and the other is thymic transplantation. These are both approaches that are being developed by our own group, and which I'll show you some more details about.

The other type of tolerance, or the other way to think of T-cell tolerance, is peripheral tolerance, in that you tolerize a T-cell after it's made its way into the peripheral immune system, and is a mature cell. Now, this has worked for some allograft models, but often not in the most stringent and difficult models. And likewise, in very difficult xenograft models, peripheral tolerance really has not been successful on its own, and it's more likely to have a role in combination with a central tolerance approach, rather than on its own.

Now I mentioned mixed hematopoietic chimerism as one of the approaches that we've been looking at to induce tolerance, and this is really based on a paradigm that we've studied in a lot of detail in an allogeneic system. And basically what it involves is treating a recipient in a way that a donor hematopoietic graft can take, so that its hematopoietic cells join the recipient hematopoietic cells in the bone marrow, recipient shown as line, and donor shown as stipple, and send progeny to the thymus where T-cells develop and get educated. And these progeny include antigen-presenting like dendritic cells that play a very important role in educating T-cells. So if you've depleted your recipient T-cells in the periphery, and then put in this bone marrow graft, you can get bone marrow cells from the donor going to the recipient thymus, and educating all the newly developing T-cells to be tolerant of the antigens presented by the donor, as well as the recipient APC's that co-exist in that thymus. So that is the basic mixed chimerism approach. And we developed some very nontoxic ways of doing this in allogeneic models, and have been attempting to apply them in xenogeneic models as well. However, doing this in a xenogeneic system is much harder than doing it in an allogeneic system. In an allogeneic system, the main immunologic barrier we have to worry about is T-cells. If we can deplete those or inactivate them, we get bone marrow engraftment.

Antibodies can limit allogeneic engraftment, but they don't pre-exist alloreactive antibodies are not normally present, and so these are obviously a greater problem in xenogeneic than allogeneic transplantation. In addition, we found that NK cells and gamma-delta cells play a much greater role. They don't play much role in allogeneic resistance at all, but they play a much greater role in resisting xenogeneic hematopoietic cell engraftment. And in other studies I'll show you natural antibodies and macrophages also limit xenogeneic marrow engraftment, and not allogeneic to any major extent.

There are also some non-immunologic barriers such as species incompatibles in cytokines and adhesion molecules and a variety of molecules that we probably haven't even thought about. And I'll show you some of those in a moment.

So overall, you can see, and we've looked at this in several different models, there is a clear competitive advantage of host hematopoiesis over a donor hematopoiesis. So to get a mixed chimerism approach to work, we need to think about a lot more things in a xenogeneic system than an allogeneic one, and this illustrates an approach that we developed some years ago that works very well in a rat-to-mouse concordant species combination. And I'm going to show you some mouse data in this talk, because I think it does illustrate some principles. It shows you what is possible if we could overcome some of these additional barriers in a large animal model, some of the barriers that you've heard about.

And this is what we call a non-myeloablative transplant. It doesn't consist of lethal irradiation or the usual toxic treatments that you have to give for a conventional bone marrow transplant. It's much gentler, it involves a low dose of total body radiation, local radiation to the thymus, and monoclonal antibodies to deplete recipient T-cells that recognize the donor, natural killer cells, and gamma-delta T-cells. And if you give a rat bone marrow graft and T-cell deplete it so it doesn't cause graft versus hostess disease, you can induce this state of mixed hematopoietic chimerism. This chimerism is indeed associated with tolerance. Skin grafting is the most stringent test of tolerance that we know of, and these mice that are chimeric will accept a skin graft from the donor, and reject a skin graft from a non-donor rat. So they're specifically tolerant of their donor by the most stringent measure. This tolerance occurs by the same central adhesional null mechanism that I referred to earlier on.

Now extending approach to a more disparate -- discordant pig-to-mouse xenogeneic model has been more difficult, and here we found that the competitive advantage of the host over the donor is even larger than in the rat-to-mouse, so even in the absence of an immune system, using an immunodeficient mouse, like an NOD scid mouse is very difficult to get pig marrow to survive. However, a few years ago, Dr. Yung Long Yang (phonetic) in our group inserted some porcine cytokine genes into these mice, making pig cytokine producing transgenic mice. And as you see, these transgenic mice very effectively support high levels of Porcine hematopoiesis in contrast to the nontransgenics, which don't. So providing Porcine cytokines can overcome some of this competitive disadvantage of the donor in a xenogeneic environment. And most remarkably, in these chimeras, Dr. Yang showed that porcine dendritic cells shown here with this stain spontaneously migrated into the recipient thymus. And this is a most important thing for tolerance induction. In fact, he's recently shown that these mice, if you then give them a mouse immunocompetent bone marrow graft, develop T-cells that are tolerant to the Porcine donor. So pig -- Tolerance to pig can be induced across a very disparate species barrier using hematopoietic transplantation. This is another study by Dr. Yang showing that in fact, even in the absence of antibody in these immunodeficient scid mice, complement in fact does limit hematopoietic cell engraftment. This may occur by some of the mechanisms that you heard about earlier this morning.

But what you see is that in SCID mice, if you treat the animals with cobra venom factor to deplete complement, you end up with higher levels of porcine engraftment than you get in control animals not getting complement inhibition.

Finally macrophages are a very important limiting factor to xenogeneic marrow engraftment. This is being shown by a Dutch group in the human-to-mouse combination. And this is another study from Dr. Yang showing it in the pig-to-mouse combination. And what you see is that, again, in immunodeficient SCID mice, now with the porcine transgenes, depleting macrophages with M-liposomes enhances the level of porcine chimerism that is seen. And this can be seen in the bone marrow, as well as the other organs. So macrophages seem to take up a lot of porcine cells in vivo in these mice through mechanisms that we don't yet understand. And this is probably very important in the pig-to-primate model as well. Our group, with Dr. Cooper and Dr. Sachs, have shown that macrophage depletion in that system can considerably increase the levels of porcine chimerism in the stem cell transplant recipients.

I just put this slide up to point out the complexity of the hematopoietic microenvironment and to make the point that in fact incompatibilities between any of these molecules on hematopoietic progenitor cells and these molecules in the hematopoietic microenvironment need to be considered as possible limitations to porcine hematopoiesis in a human environment. This is an area that we need to study more.

Okay, antibodies. They, as you know, are produced by B-cells and plasma cells. We know about natural antibodies, most are against Gal, but there are about 10 or 20 percent recognizing other specifities, and they play and great and important role in hyperacute and delayed xenograft rejection.

Induced antibodies against new specificities and against Gal are largely T-cell dependent, and can cause vascular rejection as well. So one can overcome antibody rejection by measures that you heard about: Immunoabsorption with Gal columns or infusion of gas, you heard about, and other molecules can be used. Human complement regulatory proteins help to limit hyperacute rejection, or prevent it, and then there are longer term approaches, such as induction of tolerance, or the use of Gal knockout donors. But we don't yet know whether the homozygous knockout will be viable. We are hoping it will be, or what other specificities will appear as targets of natural antibodies in a homozygous knockout. From -- Transgenic pigs with sugars that block Gal epitopes are also being considered.

I am going to say a little bit more about tolerance. Tolerance has been reported by Mark Wore's (phonetic) group in T-cell deficient rats receiving Leflunomide and infusions of donor antigen. Our own group has reported that tolerance can be induced by induction of mixed chimerism in xenogeneic and Gal knockout models. This approach, both of these approaches can tolerize to all potential specificities, including Gal on the donor. Whereas, another approach, gene therapy, and possibly the use of Gal polymers, could tolerize to Gal alone.

Now, I'm going to show you a model that we've used to examine the ability of hematopoietic chimerism to tolerize to Gal. And as you know, pigs express Gal and humans don't, and they have natural antibodies against it. And recently Hadekiota (phonetic) in the MI lab developed a model where he used Gal deficient mice, Gal knockout mice, which like humans have anti-Gal natural antibody in their serum, and looked at the ability of mixed chimerism to tolerize the Gal-producing cells. So, in this example, recently published in Transplantation, we used the rat-to-mouse model that I just told you about, this non-myeloablated bone marrow transplant model using Gal positive rat bone marrow cells given to Gal knockout mice.

What you see is that very quickly after the transplant anti-Gal antibodies disappear from the serum. So these are condition control Gal knockout mice, and these are mixed chimeras. The controls have plenty of antibody, whereas the mixed chimera show a marked reduction by two weeks, and is gone by six weeks. And this is true tolerance, because B-cells taken from these mice do not make anti-Gal antibody in contrast to cells from condition controls.

And if you put a rat heart into these mice, if you just use the conditioned Gal knockout controls that don't get a bone marrow transplant, they reject the rat hearts very quickly, within minutes to a couple of days. And here is an example of hyperacute rejection occurring within 30 minutes of vascularization; whereas, the -- and -- okay. This is -- Anyway, this is a wild type animal. None of our animals that are Gal knockout mice that receive rat BMT, none of these developed any hyperacute or delayed vascular rejection. This is a heart from one of those animals at 117 days, with normal looking histology. This is a hyperacutely rejecting graft in another condition control animal at one hour showing classical hyperacute rejection. Another animal in this group rejected later at four days, shows delayed vascular rejection. And wild type animals that don't have anti-Gal, but just get conditioning without BMT, they reject by cellular process, because their T-cells aren't tolerant. But our mixed chimeras in the Gal knockout mice have tolerant T-cells and B-cells, so all these three different forms of rejection are avoided. And this shows you the antibody complement in the control absent from the mixed chimera. And these hearts are accepted permanently, whether -- whereas a rat would be rejected in the control Gal knockout mice, and rejected within a few days in the control wild type mice. So both T-cells and B-cells can be tolerized, and all forms of rejection prevented with this approach.

I'm now going to talk a little bit about thymic transplantation as an alternative approach to inducing T-cell tolerance, if you can bear with me through the fire alarm.

DR. SALOMON: This is specially done at the 20-minute time point. Just kidding.

DR. SYKES: Am I really at 20 minutes? All right. Anyway, the -- These are normal mice, in which we've now used a different approach to inducing tolerance that is based on the mixed chimerism idea, but instead of making pig cells and grafting the marrow and going to the recipient thymus, we are now removing the recipient thymus and replacing it with a porcine thymus in mice that receive T-cell depleting antibodies. And what happens is that this pig thymus replaces the mouse thymus. It is put under the kidney capsule of the mouse, and it reconstitutes CD4 T-cells and makes them tolerant to the donor pig. And so with no immunosuppression whatsoever, pig skin grafts are accepted on these mice. This is a piece of pig skin that has been on this mouse over a hundred days, the mouse rapidly rejected a third party allograft, and by this very stringent test, the animal is tolerant. These T-cells have been centrally tolerized in the grafted pig thymus. Well, when Boris Nikolic was in the lab, he asked whether or not this could also occur for human T-cells, if we put them in a pig thymus graft now in an immunodeficient mouse. And what you see is that when you put a porcine thymus graft into a mouse, it in fact generates human T-cells, if you put in the source of human T-cells with it. I mean human hematopoietic progenitor cells with it. These are pictures of thymus sites from those grafts that are completely indistinguishable from the thymus sites that develop in a human thymus graft in a similar immunodeficient mouse. These cells populate the periphery of these immunodeficient mice. And most importantly, these human T-cells that develop in a pig thymus graft are tolerant, specifically tolerant of the pig. So human T-cells developing in a human thymus graft, they react to alloantigens in the mixed lymphocyte reaction and to pig antigens, and adding allo increases the response to pig. If you look at human T-cells developing in a pig graft from a DD type of donor pig, these are David Sacks' in-bred -- partially in-bred slide, you see that the animal, these human T-cells react to allogeneic antigens and to nondonor CC pig, but there is absolutely no response to the donor, and even when adding IL-2, there is completely unresponsiveness to the donor. These are human T-cells specifically tolerized in vivo in a porcine thymus graft to the MHC of the porcine donor.

And I am going to wrap up by saying a few words about NK cells and macrophages. So NK cells are prominent in infiltrates and xenografts undergoing acute vascular rejection, or DXR. And porcine endothelial cells can be activated by human NK cells. There is -- I mentioned their role in resisting xenogeneic marrow engraftment. They can mediate antibody dependent cell mediated cytotoxicity, so antibodies play a role in this kind of NK cell-mediated rejection.

Now human NK cells do not receive inhibitory signals from porcine MHC molecules. And I'll tell you what I mean by that in just a minute. And their killing can be inhibited by transfection of HLA antigens into porcine molecules. And just to explain that a bit further, let me show you this slide taken from an article by Moretta, et al., showing you that NK cells have a variety of receptors on their cell surface that recognize different ligands on target cells. Some of these receptors transmit positive signals that cause the NK cell to be activated, or killed. But others transmit inhibitory signals through an I-TM motif (phonetic) into the NK cell to block its ability to kill. And one of the critical types of molecules that does this are MHC molecules, HLA antigens. This is a major way by which NK cells are prevented from killing cells. But in fact, porcine cells don't have HLA, and no one has ever been able to show inhibition of an NK cell by a porcine MHC molecule. So the lack of this inhibition is very important in allowing NK cells to kill xenogeneic targets, because when people have looked at other activating receptors, they seem to work okay between species. So some activating NK cell receptors have been shown to interact with xenogeneic ligands.

Furthermore, NK cells can recognize carbohydrates. In fact, there is evidence they can adhere to and can be activated by the Gal determinant. The receptor for this is not known. So how can we overcome NK cell-mediated rejection? Immunosuppression, but that is toxic. What about tolerance? Mark Wore's group again has shown in T-cell deficient rats that administration of donor antigen with temporary NK cell depletion can lead to tolerance of the recovering NK cells, very encouraging, and we have some evidence that mixed chimerism may also induce tolerance to NK cells. And finally, transgenic pigs that express human HLA Class I antigens may be able to inhibit NK reactivity and provide a very promising approach.

So, I'm going to wrap up with a picture of a model that you already saw with -- from Dr. Zhong's -- I'm sorry, from Dr. Robson's presentation involving pig-to-porcine -- I mean pig-to-baboon transplantation in our studies by Dr. Cooper and Sachs, et al., involving a variety of immunosuppressants, including anti-CD154 to block sensitized antibody response in an effort to get Porcine mixed chimerism. Dr. Robson told you about some of the difficulties involving coagulopathy in those studies, but we think that with the advances being made, this should be possible to overcome these barriers.

And we have made considerable progress by providing porcine cytokines to these animals, and we in fact have achieved transient porcine chimerism, shown here in some of these animals receiving porcine hematopoietic cell transplants. So I'm going to -- Oh, yes, I wanted to show that. So I just wanted to end with some questions that I think need more research, and are really important in this area.

First of all, can macrophages be tolerized, for example, by induction of mixed chimerism? I've told you how important they are in DX -- in AVR and also hematopoietic cell transplantation. I'm very encouraged by the study using CD147 knockout mice, which is a receptor on macrophages involving erythrocyte recognition that gives an inhibitory signal to macrophages, and that these knockout mice, even though they could reject wild type, phagocytose, wild type erythrocytes, because they lacked the CD147 receptor, they didn't phagocytose their own, which suggests to me that macrophages can adapt to the absence of ligands, which is very encouraging.

We don't know what activating receptors they use, or which ones NK cell use to recognize porcine cells, or what inhibitory interactions may be missing between Porcine -- human NK cells and macrophages in human targets. We don't know what the critical adhesive interactions are that favor human over porcine hematopoiesis in a human marrow microenvironment, and we don't know what the critical cytokine interactions are either. So I think there is room for a lot more work, and a lot of hope. Thank you very much.

DR. SALOMON: I should start by saying that it's confirmed that this is a false alarm. I can't bring myself to make any jokes or generate speculations on why the alarm went off in the middle of Megan's talk, but you were really good about not getting flustered with that. I appreciate that.

Are there any comments or clarifications? I actually had one, Megan, I wanted to pose to you, and that is as you and I have talked before, the idea of generating T cell tolerance has got decades of background to it, and you and some others have made some real progress recently in that area. B cell tolerance has been much more difficult. One mechanism of B cell tolerance, of course, would be T cell-mediated or T cell-dependent antibody systems, but the Gal natural antibody pathways have not, to my knowledge, been T cell-mediated nor respond to immunosuppression. So can you maybe talk a little bit about why this happens and how it happens?

DR. SYKES: Right. So the model I showed you of nonmyeloablative rat-to-mouse bone marrow transplantation that tolerizes anti-Gal-producing B cells involves T cell depletion, so there are no T cells around anyway, so it can't be T cell dependent. We know that the conditioning regimen, the relatively mild nonmyeloablative conditioning regimen we use, doesn't deplete B cells either. And in fact, condition-controlled animals show an increase in natural antibody levels, not a decrease. So we know that we're tolerizing preexisting B cells in these animals, and we are also tolerizing newly developing B cells. And what we've done is used a Gal polymer to look at the fate of anti-Gal-producing B cells in these animals, and what we've found is that in the long-term chimeras there's an absence of B cells in the animals bearing receptors that recognize Gal. So that's consistent with either clonal deletion or receptor editing of newly developing B cells. But the tolerization of the preexisting B cells is a bigger mystery because there we can see at two weeks after transplantation when there is tolerance, we can detect the anti-Gal receptor-bearing cells in the mice. And we've done some adoptive transfer studies that we haven't published yet where we have found that in fact the early mechanism of tolerance requires a continued presence of antigen, so it looks like it's some kind of anergy that may be dependent on cross-linking by the cell-bound Gal on the donor cells.

DR. SALOMON: Dr. Paulson?

DR. PAULSON: You showed some studies with the Gal knockout mice, and I think we can assume that the pigs will be viable since the mice are viable. It's a similar knockout.

DR. SYKES: We hope.

DR. PAULSON: So assuming that that hole is plugged, using Dr. Zhong's analogy, but Jeff Platt's concept of a few factors being important, when you tolerized those mice to T cells, you had a very good result with respect to lack of rejection. Do you think that those two would be sufficient?

DR. SYKES: I hope so because the thymic transplantation approach seems very effective at tolerizing T cells. And I didn't have a chance to show some exciting studies being done in our research center by Kozi Yamada and David Sachs with thymic transplantation in the pig-to-baboon combination, which seems to have activity in tolerizing T cells as it does in the mice. So in that case we wouldn't need to worry about hematopoietic engraftment if we could use thymic transplantation in combination with a Gal knockout donor, but we don't have the donor yet, so we don't know whether that will work in the pig to primate, but that is our hope. I should say though that 10 to 20 percent of anti-pig natural antibodies are against other specificities, and I don't think we can be confident yet that those may not create considerable problems with a vascularized xenograft.

DR. SALOMON: Okay. Dr. Vanderpool?

DR. VANDERPOOL: Megan, could you just briefly put in perspective the relationship between tolerance induction and some of the other approaches? You see these at this point as all necessary for organ acceptance or do you think it's possible for tolerance, for example, to be the dominant solution?

DR. SYKES: I see tolerance as, in my view, the best long-term solution. Tolerance can take care of the T cell response. It can take care of the antibody response. We think it can take care of the NK cell response. And we don't know if it can take care of the macrophage response. But then there are these other elements, like coagulation abnormalities. Can those adapt? We don't know how much of the complement cascade could ever adapt. So in my view we are going to need genetically modified pigs. To what extent they need to be genetically modified we don't yet know. I suspect that we're going to want some additional genetic modifications, for example, human HLA molecule expression or human adhesion molecules or human cytokine receptor molecules, but I think we'll want some additional transgenic pigs, and I think the transgenes that already exist in overcoming the problems we heard about this morning are likely to be important in their own right in order to allow tolerance to occur in the first place and in order to allow the grafts to avoid destruction by other mechanisms.

DR. VANDERPOOL: Thank you.

DR. SALOMON: Okay. Then I'm going to turn this back to Megan, and the next phase of this is a panel discussion.

Agenda Item: Panel Discussion with Speakers and SACX Members

DR. SYKES: All right. So at this point I would like to start. I think Harold's last question actually allowed a little bit of a summary of what we heard this morning and how it all ties in together. So I think we should start by just opening up the floor for questions on any of the talks that we've heard this morning.

DR. SWINDLE: I have a general question. Since we started with these various conferences in about '95, the prediction has always been in five years we'll be doing clinical transplants, and I notice in the paperwork here we've still got the same five-year figure. So a two-part question to that. Is there any reason to believe, even with the progress that's been made, that this five-year figure is any more realistic than the one seven years ago? And secondly, in terms of whole organ transplantation, let's say we get the cellular transplants to work in five years, but with whole organ is there any reason to believe in the foreseeable future that that could get any further than just a bridging type of operation to wait for a human donor because of the long-term tolerance issues which haven't gotten beyond the acute rejection phenomena yet? So it's an esoteric question, but I'd just like to hear the predictability on where you think this is going.

DR. SYKES: Volunteers to take that on? Dr. Platt?

DR. PLATT: Well, when will xenotransplantation enter the clinical arena? It's in the clinical area. As your question implied, there are experimental xenografts of various sorts being carried out, and whether or not they're successful is really a clinical question at this point more than it is a biological question. So it seems more the question of the moment is when will organ transplants be carried out? And I think there the salient issue again is a clinical one. No one wants to do a transplant that is not going to benefit the patient. And so assuming all of the other aspects of this have been settled in one way or another, one has to look for the appropriate setting in which to introduce a transplant so that the patient can potentially benefit. As I see it in the future, xenotransplantation will be part of a number of modalities that can potentially be applied, but the time that it takes to apply new modalities for organ replacement in part reflects the care and the caution that clinical practitioners apply, and that's part of the art of medicine.

Nobody wants to hurt a patient. Everybody wants to help a patient. Could a xenograft be done today? Well, possibly. We just don't know. But nobody's willing to do it today under the present circumstances unless there's a clear opportunity to benefit the patient.

DR. SYKES: Thank you. Anyone want to add any further comments to that? Dr. Vanderpool?

DR. VANDERPOOL: I appreciate Dr. Platt's comments. There may be populations of patients out there right now who have no chance of an allograft who could be benefited, and that's a tough question. I remember debating this with different people taking different sides in the FDA subcommittee on xenotransplantation two or three years ago. I think one question I have on the way of thinking about answering that is to return to Dr. Zhong to maybe put you on the hot seat a little bit. I'm not putting you on the hot seat, but on the soft seat. We've got humagenic pigs working with baboons, and so that's not necessarily the successful model, given the degrees to which clinical interventions are designed for human beings, and we're dealing with humagenic animals and not primagenic animals. So would it be possible for xenotransplanted organs to be significantly more successful than the primate models? I mean that's one question at least to ask. And if so, how soon would that be a viable clinical alternative? I do think there's an ethical question involving future possibilities. One of the things I argued in an article not long ago is I think one of the challenges we have is to do it right the next time around as much as possible because every time mistakes occur, you get set back pretty seriously. So what that equation is to do it right is really part of what I think Dr. Platt may be referring to. But back to you. I mean if you're dealing with primate models, to what degree would human clinical organ transplants be more successful than the things that are occurring with primates at the present time?

DR. ZHONG: Well, it's a very good question. I would like to take the example of looking at early animal transplant experience. When we develop some new drug, like cyclosporine or FK, we always test in the monkey or baboon model. At that time if you looked at the literature on survival, no one can achieve a one-year, two-year survival. As long as we can achieve a reasonable long-term survival at that time, actually we moved to the clinic. So my view is that I was told the FDA set a minimum standard for us to start a clinical trial that is the 100-day survival. 50 percent of the baboons need to live for 100 days with a normal function. We have not reached to this milestone yet, but I think we're a step closer. So my problem is that, first of all, we all know there's a severe shortage with organs, but the current problem I'm now having is some of the industry have left, and we really have a limited resource -- particularly I talk about Canada -- to do nonhuman primate research. And also there's a problem that there's a lot of good technology available, for example, knockout pig in the future, some good drug like anti-CD40 ligands, but the problem is all these technologies currently are controlled by some industry. So I think if the NIH is able to put all the resources and available technology together, I think we can move this field much faster. One of the examples -- and I think Steve Rosas is here -- in allotransplant NIH did a wonderful job to have a tolerance network. In this network we worked together, including myself. We just had step-to-step working of the nonhuman primates, and I think we had very good progress. I hope if NIH can do a similar organization, I think this will be the way to achieve the success.

DR. SYKES: I would just like to reinforce that point. I think the answer to your question, Dr. Swindle, is really the speed with which we get to the ultimate goal of xenogeneic organ transplantation is going to be very dependent on the amount of support given to the field and the infusion of people to look at these many, many questions that have not yet been answered. Every single speaker this morning talked about what is known in this field and also pointed out areas of major, major unknowns, and every single speaker had ideas of what we need to look at and how we could overcome these problems, but there is really a very small pool of investigators working in this area, and I think the speed with which we get to our goal will not be limited by the creativity or the technology that's in existence, but more by the resources that are put into it. Dr. Collins?

DR. COLLINS: Thanks, Dr. Sykes. Dr. Zhong, I'd like you to take your comments a little bit further. First of all, I have great respect for anyone who does baboon research. They're about as cooperative as my three-year-old, not very. When you look at the first organ transplant or xenotransplant, do you see subjecting a renal failure patient or a diabetic to a whole organ transplant where there's an alternative therapy, dialysis and insulin, or do you think that it will be limited to a life-saving transplant, such as heart or liver?

DR. ZHONG: Well, this is a good question. We're always debating which organ should we start first. The advantage for kidneys is if anything goes wrong, we can put the patient back to the dialysis. But again you're absolutely right. The kidney transplant is not a life-saving procedure. However, for some patients who were presensitized, they were not able to receive the human donor and some patients who had severe complications due to the dialysis. So in these patients xenotransplantation may be the option. In the heart certainly you're absolutely right. We are facing a very severe shortage of hearts, particularly for pediatric patients. So for these kids they're dying, so the xenotransplantation may be a solution. And also I would like to emphasize using the pig organ as a bridge, like extracorporeal perfusion, also is a very attractive way. Dr. Levy will talk about that subject this afternoon.

DR. SYKES: Dr. Bloom?

DR. BLOOM: I just wanted to comment on the notion that FDA has set a 100-day survival in baboons as a hallmark for allowing clinical organ xenotransplantation to go forward. I'm completely unaware of that, and I probably would know about it. The discussion that Dr. Vanderpool referred to at an FDA advisory committee a few years back -- and a number of people here were at that committee meeting -- discussed at length what types of things FDA should look for before allowing whole organ xenotransplantation to go forward, and in fact, time in a preclinical trial was one of them. I don't recall a specific time point being said. I do remember something like six months being bandied about, but there is no set time that we are looking for. What we are looking for will be a prolonged survival sufficient to justify using humans, and then it would be likely that we would take that question to an advisory committee such as this one before it would be allowed to go forward in any case. That committee also discussed ideas that you just put forth about whether a heart transplant should be first perhaps because that could be life-saving, but on the other hand if it fails, that person has no place to go. A kidney transplant, because it may not be life-saving, but if it fails, that person does have something to keep them alive perhaps until an allotransplant could be available. So these are major questions for us that we do not have the answers to.

DR. SYKES: Dr. Robson, I think you were next.

DR. ROBSON: Just to echo all those other comments, the main rationale for xenotransplantation is the shortage of allografts, and extrapolating from the current experimental data of 60-day survival, we would only be using these as a bridge. And with regard to kidney support, if the patient has vascular access, then there is the rationale for dialysis. For cardiac support there is the artificial heart. For liver support there is, as Dr. Levy's going to be presenting, some bioartificial support which can give short-term benefit. But I think what we'd be doing by using this as a bridge is also exacerbating an organ shortage. And just to echo another point of Dr. Platt, I think our main function as doctors is also to do no harm. So we're not only providing a bridge, but we have to be absolutely sure that that organ is not going to be doing any harm to the patient. And from my perspective, that revolves around calculation disturbances and introduction of stroke and so forth. So I would like to obviously be a bit more conservative and make sure that we're not endangering the patients by using these as bridges.

DR. SYKES: Mr. Berger?

MR. BERGER: I'd like to add on to Michael's question before. It seems like every day we've been reading articles and seeing advances, but I've followed xenotransplantation for the same seven years. It's the same thing. It's coming. It seems to be going on a little bit slower last session, and I'll be careful to comment on the public summary that Eda did about human clinical trials. It didn't seem like a very good success record in summary. Everyone here has asked for more research, that we need more research in certain areas. So I have a two-part question. The first part, which is one of the things that's been recommended to this committee, number one, is xenotransplantation losing ground to things that are outside of xenotransplantation, whether it's medical devices or stem cell or tissue engineering, and should we be comparing the progress with xenotransplant to things that are going on outside it? And number two, which was something that Dr. Platt brought up, in looking at organ transplants, separating them per cell and tissue, is money and research being divided in too broad of a sense rather than concentrating in an area that you're showing may be easier per se?

DR. SYKES: Dr. Platt?

DR. PLATT: Those are very good questions. I would like to address a few of them to the extent that I can do so with any authority. Unfortunately I think no one person can answer the questions that you've raised with authority. As far as comparing xenotransplantation with other modalities is concerned, I think this is a very important and evolving consideration, and hopefully it will always be evolving because we will always be improving not only the prospects for xenotransplantation, but the prospects for these other modalities. I would point out, however, that the first xenograft, as far as we know, was attempted in about 1906, so we have nearly 100 years of experience in knowing what happens when an organ is xenografted. We know a great deal about it. The very elegant presentations this morning really detailed what we understand about many of the problems and potential solutions. We know much less about some of the other modalities, such as stem cells. It's a wonderful word to put out there. There's a lot of excitement and a lot of possibility, but we just do not know to which extent these other things can, now or in the future, replace organ transplantation as a way of treating disease. So I think we have to temper our enthusiasm with a relative understanding of what the limitations are, and we know a lot more about this than we do about other fields.

DR. SYKES: Dr. Scheckler?

DR. SCHECKLER: Let me try to pull some thoughts together here as a nonexpert in any of these fields. The first comment I would make is do we need to know all that might be knowable before we know enough? It sounds like that in the area of complement or platelets or endothelium, that once you get some answers, you have 35 more questions to every answer that you come up with. And I don't know, in the area of infectious diseases, for example, the whole porcine endogenous retrovirus and the concerns about that, that we'll ever know everything or know enough. So that's sort of the philosophical underpinning about when do you start to do clinical trials with some of these things? It was pointed out at our last meeting that we as taxpayers have been supporting the quest for an artificial heart since 1963, '64, and we're putting artificial hearts in folks probably with less concern than we're using for a xeno knockout pig heart in terms of what are the backups. There haven't been much, and those folks have died, and they've had strokes and all kinds of problems, and I'm not sure all that is knowable about artificial hearts before they're put in is the question that's asked. So to get to my point, and this has sort of been talked about a little bit here. I had a professor that once told me that a kidney is a smart organ, and a heart is a dumb organ, that there aren't too many things that the heart does compared to say the kidney or the liver. Should there not be a focus on the dumb organ first, on the heart that has much less complexity in terms of the kinds of filters in manufacturing that it has to do versus the liver or the kidney? That may be a simplistic question to you all, but it seems to me to be a perfectly rational question. And can't we move forward with one of these organs first without trying to know everything about everything before we do anything?

DR. SYKES: Dr. Platt?

DR. PLATT: I'd like to take a stab at that one, and then perhaps I'll be silent for a bit. I think the question that you're raising is a very appropriate one about the relative considerations of the heart and the kidney, but it happens that the heart has a peculiar vulnerability that the kidney and the other organs do not, and that is that when coagulation occurs inside of a blood vessel of the heart, it can cease the function of the entire organ. An example of that is when somebody has a heart attack and their heart just stops. In the case of the kidney, if you form a blood clot in one blood vessel, the kidney can just go on and function normally. And in fact, you'd have to have that occur in 90 percent of the blood vessels to the kidney in order to have no function or little useful function from the organ. So there are reasons to focus on organs other than the heart potentially for xenotransplantation that relate to the feasibility and the potential well-being of the patient. There are reasons to support looking at the heart, but I think that there are reasons to focus on some of the smarter organs as you call them.

DR. SYKES: Dr. Robson.

DR. ROBSON: Chris Barnard, who transplanted the first human heart, used to say, the heart is a pump. He also used to say, you have to make a choice. You can't marry all the pretty girls. But there again, it's Chris Barnard. The heart is unique in certain aspects with regard to endothelium. It's much more sensitive to the use of fibrinolytic drugs. And we know that great success has been had with urokinase TPA in unblocking the arteries of the heart. The kidney has much more complicated regulation of clotting on the endothelium. If there's any kidney damage, the kidney leaks proteins from the plasma which are protective against clotting. So I would think from that aspect that the heart would be a better option for an acute bridging experiment. And your point is quite correct about the actual concern about transplanting artificial hearts with regard also to the concern we have about transplanting porcine-modified xenografts. There are other obvious considerations with regard to zoonoses and so forth that we probably will address later.

DR. SYKES: Dr. Swindle?

DR. SWINDLE: Yes. Actually a clarification from Dr. Vanderpool. I was just reading the charge of the committee here, and a combination of Alan's comments and Megan's comments struck me here. Is it within the charge of the committee in your opinion for us to make recommendations on funding future directions to the Secretary? Is that the way I read the last charge of the committee? Is that within our purview to say, after we weigh everything, that this problem could be fixed if more money was put into this direction versus another?

DR. VANDERPOOL: Mike, I'm going to have to read that part of the charter. I've been working on the charter change. See, again I do think that the science working group might consider as one of its concerns, if it wishes to, to bring to the committee a recommendation that greater funding be given to these scientific areas so that we can move forward. My response to Alan Berger is that stem cells are way off in terms of creating organs. Our talking about artificial organs at this point needs to wait until Dr. Michler's comments, who just arrived and who will be talking this afternoon. So our doing those comparisons probably ought to wait. But it seems to me that one of the things this committee should do is that if indeed we judge this to be a modality that has great promise, we should press for funding because without it we can't move forward. I mean one of my overarching feelings so far today is that a lot is going on, and it's good to hear what's going on, and I'd like to see some of these developments pursued with all due speed. I'm not answering your question, Dr. Swindle, but I will look at that charter closely and try to get a better feel. But I think we should feel empowered to make recommendations to the Secretary regarding any sets of issues, including better funding.

DR. SYKES: Dr. Levy, you had a comment.

DR. LEVY: Yeah. I was just intrigued by the question. I run a major multi-organ transplant program north of the border. First of all, I think the issue with when is the timing right for xeno, I'm not sure there is an answer. I think if you took a survey around the table, you'd probably get a whole series of different answers. We went through this exercise in Canada, as you're all probably aware, and I think part of the reason why there isn't an absolute answer, coming back to perhaps the questions that you're asking, is it's one thing if it's a personal benefit for an individual, and it has no impact on a society, and this I think was brought forward. It's another thing when there's a personal benefit, and it has a societal impact. I think that's what everybody is struggling with as to what is the risk-benefit if it doesn't succeed, but the person lives, for instance, and ends up on some form of other modality. And I don't think you really should compare xeno to an artificial system. I'll talk about extracorporeal liver systems. Dr. Michler will talk about heart systems. I don't think they're necessarily comparable, especially when you talk about innate objects where there may not be the risk. I know Dan didn't want us to get into the zoonosis issue, but where there is risk to society, at least it has to become part of the public discussion.

I also want to make another comment. There is no such thing as a dumb organ. None of our organs are dumb. They're all intelligent, as we are I hope. The issue is a heart, for instance, isn't just a pump because we know it makes hormones. Atrial naturetic factor is one. It makes other hormones which control sympathetic tone. For instance, if it just beat at a fixed rate, that wouldn't be very helpful to many of us who want to do certain things. Like I jogged this morning. Just having a fixed rate instrument wouldn't necessarily help me. It may not help me anyways, but that's another issue.

And then the final comment I make is in coming back to the funding. First of all, there's been tremendous progress in xeno. There are xeno trials going on, as you know. There's cell transplant trials that are going on. There's extracorporeal trials that are going on. There have been solid organ experiences, and I think the work that Megan has done, that we did, that Bob did and so forth, I think that no one 20 years ago would have thought that we would have gotten a primate to 75 days. I don't think Jeff, who's not been in it for 75 years, but obviously no one who was involved would have thought we would have gotten that far. So I think there is a lot of progress.

The final comment I want to make is I don't believe, just looking at the field -- and I sit on a government stem cell committee. There's been a lot of hype and enthusiasm with stem cells too. And in our country the committee made a recommendation to tone down the hype because there was a lot where they said we would be in clinical trials, and we'd be generating hearts, and we'd be replacing in vitro organogenesis hearts, and they likened our committee to what had happened in xeno. I think a lot of what you're struggling with is you said whoever made the comment it's five years and then five years and then five years and so forth. But as a transplant scientist and a transplant biologist, I don't believe there will be one solution for transplantation. I think there will be many solutions. I'm going to allude to that with liver disease because I think it depends on the type of disease, the stage of the disease. Prevention is best. If we can't do that, then we have to look at the different stages and when we would go in. Someone who has end stage disease, putting stem cells in may not solve their problem. Someone who has acute disease in which we can regenerate or refurbish the organ, that might be a good solution.

DR. SYKES: Thank you. Dr. Salomon had some comments.

DR. SALOMON: I wanted to try and veer back a little bit to the basic science theme of the morning. What I find really interesting and natural is when scientists go at the molecular bases of these events so that we're looking very specifically at elements of the complement system, regulation of carbohydrates, regulation of clotting system, fibrinolysis et cetera, it can almost get overwhelming. Hundreds of targets

come up, and you look at it, and you go, okay, I give up. And then you come around, and you put a pig kidney in a baboon, and it worked for 73 days. Now, there were problems there, and that's not my point, but the fact is that the primate studies, and to some extent the mouse studies that preceded them, put this into an interesting dynamic. So the question I have for the panel is can we think of ways, can we think of better strategies to figure out what are the important molecular elements by creating sort of a dance between the larger animal studies and these basic one by one molecular-driven studies?

DR. SYKES: Dr. Dalmasso?

DR. DALMASSO: Being one who has contributed to the details this morning perhaps in an excessive way, I think that's a very good question. I think, also coming back to some of the other issues of what was discussed before, that there probably have been very important strides in xenotransplantation, but I think that we are still faced with a number of very important barriers, acute vascular rejection being the most prominent. In my mind at least it makes me think twice about being so rushed to move into clinical trials. I think that the pressures from various sources, just to mention one industry, sometimes are a little excessive. I think we need to have patience, and I don't think that we need to know everything. I don't think we need to know every little detail of mechanisms, but I think minimally we need to be able to overcome barriers such as acute vascular rejection. It seems to me that it's dangerous to talk about when we are going to go to clinical trials. I think that we also have to consider the negative impact that trials that will result in bad experiences will have on the public and the support. I think that the scientific community working in xenotransplantation is very small compared to other areas, and it's important to attract more people to the field that bring in basic science to help solve the problems that need to be solved and to develop some level of patience.

DR. SYKES: Thank you for those comments. I must say I'm very much in agreement with that approach. I think patience is really what we need in this situation.

I have a question for you, Dr. Dalmasso, related to a rather cryptic comment that you made during your presentation, which is you were not certain whether there was an advantage to human complement regulatory proteins as opposed to porcine proteins or that species incompatibilities were not necessarily as important as was thought. Could you expand on that?

DR. DALMASSO: Well, what has happened is that the phenomenon of regulation of complement activation by DAF and some of the other proteins has classically been considered as being highly species specific, but in the complement system, like in the coagulation system, many times you can replace one protein or one component from one species into another species, and things move along very fine. With the complement regulators, in reality something similar happens. When we proposed the idea of using human DAF and MCP to prepare transgenic pigs, we went on the knowledge at the time of species restriction. And I think that that continues being true. However, more recently it has been found that pig CD59 and NCP, as well as DAF, are very effective to inhibit human complement. And because the human complement inhibitors also function as receptors for certain pathogens, for example, membrane cofactor protein is a receptor for the measles virus, and DAF is a receptor for certain viruses, several viruses of the Coxsackie and viral family, then the reasoning was why not overexpress the naturally occurring regulators of the pig? So this is something that is now under consideration. The experiments have been done mostly in vitro, however, there are some ex vivo perfusion experiments using pig inhibitors that show that they work. However, from there to moving to the pig and trying to overexpress that in the pig, it is going to take some time and effort and money. On the other hand, using pig inhibitors instead of human, we are going to add more antigens. These molecules are very antigenic. So we may create another problem. I think it's an area that needs to be investigated, but I don't think that it's a high priority at all because as it is now, we have excellent transgenic pigs that express human inhibitors that function very well.

DR. SYKES: So overall do you think that the benefit of the human inhibitors that have been put into pigs has been due to the total amount of complement regulatory protein express, that it's simply a quantitative effect?

DR. DALMASSO: It's felt that way, yes.

DR. SYKES: I see. I mean would you think that having overexpression of a porcine regulatory protein in a pig might in some way put that pig at risk? Would it have inadequate ability to activate complement, or do you think that since the human one doesn't do that, the pig one wouldn't either?

DR. DALMASSO: Again it depends. If it is inhibiting complement in the middle of the reaction as we do with these inhibitors, most likely not. But in relation to that I wish to make a comment regarding other inhibitors. If we want to try to inhibit complement activation very early at the level of C1q, then that really may create a problem for the donor, and if we do it systemically, certainly for the recipient of the graft.

DR. SYKES: Dr. Salomon, you have a comment.

DR. SALOMON: Yeah. A question back to accommodation. Certainly either you suppress the complement activation forever or you get tolerance or you get accommodation. So just to focus on accommodation for a minute, can you guys comment a little bit more about that? That just wasn't satisfying to me. What is accommodation? Why are we talking about accommodation? Because if it's antibody-mediated in retrospect to the ABO studies that Dr. Mendez mentioned, we can get rid of the antibodies, and we're not getting accommodation because these animals are getting rejection. So why are we even still talking about it? Because it was mentioned by two different speakers. So can we talk a little bit about sort of what's accommodation, what are you going to do to make accommodation work better, and why it's not working now?

DR. PLATT: Well, accommodation is the apparent acquired resistance of an organ to humoral-mediated injury. As I mentioned, it was first discovered in studying ABO incompatible transplants in humans, which for some period of time were thought to be impossible, were very difficult to undertake, but it was found that if you made a temporary manipulation of the recipient, removing the antibodies, that the organ would continue to function and function as well as an organ that's ABO compatible even when antibodies return that are directed against that antigen. As I suggested in my brief lecture, I think that there are lessons from this that are quite applicable to xenotransplantation. I only gave you half of a lesson before, and that was that the antibody/antigen system as far as we can tell is analogous to the anti-Gal/Gal system in terms of the amounts of antibody, the quantity of antigen, the consequences of antibody binding and so forth. What this suggests is, first of all, that accommodation is a real process. We know that it is from studying humans, and we know that it is from studying animals. But it also teaches us that if one simply eliminates the source of antibody binding, or it allows us to infer that if one simply eliminates the source of antibody binding, that in the case of xenotransplantation one does not necessarily solve the problem because, as your question then suggested, in ABO incompatible grafts which accommodate, the grafts do perfectly fine and function in perpetuity, whereas in xenografts when all of the things are done that it takes to induce accommodation of an allograft, and when incompatibility, which I do think exists in the complement system, is to a certain extent controlled, you don't have a graft functioning in perpetuity. So this suggests that there is a further barrier whether it is antibodies against other antigens or some reason that accommodation can't be achieved as well on a xenograft as it can on an allograft, but I think it's essential to focus on the in vivo models. As a scientist I'm quite vested in focusing on in vitro questions, and I think the failure of grafts has taught us an enormous amount, and we owe much of our knowledge of immunology to what's been learned from the failure of grafts, but for a practical application like

xenotransplantation or allotransplantation, we have to focus on what will work. We know that accommodation under certain circumstances will work, and if we can make it work in xenotransplantation, then that means we can probably not have to deal with many individual molecular problems that we can identify.

DR. SALOMON: Do we have a sense for what is accommodation besides this phenotype?

DR. PLATT: No, I don't think we can say accommodation is this at a molecular level because every this that has been tested has been insufficient. But I think what accommodation has taught me -- and perhaps this was obvious to some of you before -- is that every biological reaction has an opposing or controlling aspect to it. The complement system wasn't made just to destroy our own cells. When you activate complement on cells, they acquire resistance to complement. When you activate T cells directed against a target, they acquire resistance to T cells. So I think that accommodation is part of a broader phenomenon that our cells are much more adaptive than all of the figures and diagrams we show up here gives credit to.

DR. SYKES: Dr. Chapman, did you have a question?

DR. CHAPMAN: We heard a lot about a variety of things that can go wrong when you attempt xenotransplantation this morning and a lot about intellectual approaches or scientific approaches to try to address those problems. There have been some questions trying to get a comparative sense of where xenotransplantation as a solution to organ failure might rank relative to alternatives and some questions about specific approaches. I listed six approaches to trying to overcome these problems that I pulled out of the talks this morning, and I'm wondering if it would be worthwhile for the committee to kind of go through those one by one and have the panel try to give us a more concrete sense of where solutions stand on that along the spectrum from great untested idea to available in the clinic for trials. I mean the first thing that I heard people talking about was attempts to change the donors of organs or tissues in ways that decrease the mismatch with humans, and in that way, to prevent some of these problems. One approach we heard as a solution to that was the development of transgenic pigs, and I notice we have three talks on that this afternoon, so I don't think it's really worth going into that detail. Were there other approaches to changing the donor that were talked about or were not talked about that the committee members should be aware of?

DR. SALOMON: I mean one follow-up would be to put this to Dr. Paulson. You focused nicely on how the carbohydrate system, the siglec and the galectins, et cetera, would affect interactions for host immunity. Can you suggest strategies that could be allowed on the donor side, on the pig side, that might manipulate or positively affect interactions with that system?

DR. PAULSON: Well, the short answer is no, I don't think there's enough known yet about the human side, the host side, to rationally suggest that there might be differences on the donor side to change the donors right now.

DR. CHAPMAN: There's one other thing I'm aware of that didn't come up here, in part, probably because it's infectious disease related, but there have been studies coming out of several labs looking at susceptibility of expression of PERV from pig cells to antivirals that were developed to treat HIV infection, and at least AZT seems to have some potential suppressive ability, so that might be a way you could treat pigs and suppress to produce this. The second thing that I had drawn out of all of your talks was rather than try to change the donor, attempts to remove things from the human recipient that trigger these problems, sort of filter things out. And we have an example that works in another field. We know since the '60s we've had fine routine success in taking people with kidney failure and dialyzing them, which is nothing more or less than filtering their blood periodically to remove things that we don't want

there. There was at least one example that you referred to, Dr. Robson, of efforts at filtering out these naturally occurring antibodies before the xenograft receipt. Are there other examples of ways that this approach has had some success?

DR. ROBSON: I think your question is with regards to the coagulation system. Certainly the complement activation can trigger coagulation, so removing complement by cobra venom factor, by plasmapheresis, the removal of natural antibody, the suppression of elicited antibodies, all of these will prevent endothelial cell activation, regulation of pro-coagulants. These are important because the deficiencies in the cross-species, molecular incompatibilities and so forth, are not absolute. They're relative. I think an analogy would be with human disease. For example, a patient with antithrombin 3 deficiency or homocystinuria, homocystinemia, may not have any problems with thrombosis until their vasculature is stressed, or they sit on an airplane for 12 hours and end up with a thrombosis in their thigh veins. So potentially when you're stressing endothelium of such a thrombophilic vasculature, that would lead to the coagulation manifestations. And that goes back to one of Dan's questions. How come you can get to 75 days without coagulation problems? And again these are relative deficiencies. Presumably in that one animal there wasn't that same degree of endothelial cell activation, there may not have been porcine cytomegaloviruses. All of these factors are important in dictating systemic complications. Suffice it to say all the grafts that are rejected end up with fibrin, so there is a local manifestation of that, but certainly removing antibody and removing complement, part of the immunoabsorption techniques remove fibrinogen, remove clotting factors, so that could also prolong the actual survival of that xenograft.

DR. CHAPMAN: And all these things you're listing are things that have actually shown some at least small effect in a clinical trial in animals?

DR. ROBSON: Sure.

DR. CHAPMAN: Okay. Another approach that was talked about was adding something to the host to smooth out these problems. Dr. Paulson mentioned something that's actually in phase three clinical trials, which is one step away from actually being sent back to the drawing board or being approved for availability in drugstores. Are there other drug therapies that are under development, and what stage are they in? Dr. Zhong talked about making a choice in the immunosuppressants used between two, based in part, that one tended to increase clotting problems, and the other tended to decrease clotting problems, so he went with the drug that decreased clotting problems since clotting is already a problem here. Are there other drug therapies practically that have shown progress in clinical trials?

DR. ROBSON: If I can add another penny's worth. Cyclophosphamide, which we use as an immunosuppressant, we had a reductionist approach to seeing this as affecting leukocyte numbers, immune responses. When we look at it in more detail, we find that it actually affects platelet function dramatically, not just by dropping and causing thrombocytopenia, but by qualitatively affecting platelet function. So some of the benefits that we see with cyclophosphamide and other groups, where there may be no coagulation problems, may reflect effects unrecognized to date on platelets and coagulation.

DR. CHAPMAN: Another thing that was mentioned was moving from whole organs to cells because Dr. Platt pointed out that it's often the vascular component of the graft that induces a lot of these clotting problems. And again that's going to be talked about this afternoon, so I don't really want to ask about it now. There's this question of accommodation. I think Dan's already sort of fished out a little bit about where that stands in terms of practical application. Sounds like there is a model for that being developed in allotransplantation with ABO incompatible organs that hasn't yet been translated into success with xenotransplantation.

DR. SALOMON: Well, my comment on accommodation would be that maybe the target for examination ought to be the pig endothelial cell, for example, since one of the things that we have to acknowledge is that maybe it's human endothelial cells are able to accommodate, but pig endothelial cells are not. And if that were true, there are molecular strategies that we could get at to look at the genetic programs in these cells and perhaps fish out a gene or two that was missing in the pig that might allow accommodation to occur.

DR. DALMASSO: In regard to that suggestion, there are several labs that I include on my own that have taken that approach, trying to induce accommodation in vitro and then in organs by using strategies that result in two recognized mechanisms that underlie accommodation. One is the activation of protective genes, anti-apoptotics, anti-oxidants, and so forth, and then another one is to induce TH2 predominance in the type of cellular response that one needs to generate to achieve accommodation. So those things are ongoing. I think that accommodation has been extremely successful in the ABO model in humans. I think it's been very successful and very well studied in rodent models. And now I think that with Jeff Platt's preliminary or not so preliminary work moving into the primate, I think that that is one of the major areas to continue working.

DR. CHAPMAN: The last major approach the speakers pointed out this morning was immune tolerance. I know that is an area that NIH has put a lot of funding into, not just for xenotransplantation, but more so for allotransplantation and other clinical entities. But Megan, could you give us a little sense more concretely of where things stand in the progress from great idea to actually something ready to go into --

DR. SYKES: Well, we have demonstrated that human T cells can be tolerized to porcine xeno antigens by thymic transplantation in a murine model. We've demonstrated that murine T cells can be tolerized to porcine antigens by induction of mixed chimerism. I think those are two important proofs of principles that say to me that if we could successfully engraft porcine bone marrow or thymus into a nonhuman primate, we would induce tolerance. We have some preliminary data supporting that in a primate model. So in my view we have the critical proofs of principle from the murine model, and what we really need now are to overcome these other problems so that we can apply this in the primate model. So I think the limitations involving the natural antibodies, the coagulation disorder and so on, have limited us thus far, but that as progress is made in these other areas, we will be more readily able to apply what we've learned is true in principle to a primate model.

DR. CHAPMAN: So you've been able to do it in a mouse? You've been able to induce tolerance in a mouse where it will accept pig tissue by getting a mixed pig-mouse bone marrow?

DR. SYKES: Yes.

DR. CHAPMAN: And you've been able to do it in the human T cell, which is one of the main components of this rejection, when you take that T cell out of the human environment and put it into the mouse environment, so in sort of a special laboratory? You've been able to induce it in this one component of the human?

DR. SYKES: Right. Harold?

DR. VANDERPOOL: I just wanted to back up a little bit. You know, the various approaches to the increasing organ shortage, some latest figures say that about 5, 000 people more a year, 5, 000 more Americans every year, are dying for lack of available organs. We do have a crisis on our hands, and there are several approaches to try and revolve it, get more organ donations, which Tommy Thompson is wanting to do, but which is difficult, or artificial organs, xeno, and down the line, stem cell research, and just not doing anything about it because we're having to manipulate animals, cross natural barriers, and

various people take that position. I think our orientation really just touches two of these versus what about xeno and what about artificial organs. And we are going to hear about that, but our focus obviously is on xeno. Now, within that our charge is to advise the department on the current state of knowledge regarding xenotransplantation. That could be sort of a neutral thing. Well, here's the latest thing we know about workable solutions, and here's what we know about infectious disease worries. Those are the two main areas of the science. Seems to me within the workable solutions we do have a challenge to pick up, as Dr. Chapman has been pressing us to do, to think about what the solutions are and which ones seem to offer the greatest promise. I don't think we need to put any dates on any of this, but which ones, if appropriately funded, really will enable us to move forward and reach a stage of clinical organ transplants? And that's the challenge of the scientific working group. The good thing is to hear the different approaches, and it's probably easier to know what's being done than to be able to forecast how far along we are and how much more is needed in order for these to be all the more promising, but that seems to be what we need to do in order to make the kind of report that we need to make as a committee. I mean the science group can bring to us what you wish in terms of what all you want your statement to be, but I think it's one thing to summarize where are we scientifically and another to do at least some degree of forecasting regarding where we need to go from here and what it will take to get there with respect to, for example, funding priorities.

DR. SYKES: Thank you. I have a question for Dr. Platt. Can you give us your sense of what would be the most promising approach to avoiding endothelial cell activation and destruction as far as modifying a donor pig goes? Do you think anti-apoptotic proteins are going to be critical or is there something else that you would place your bets on?

DR. PLATT: I have two rules of genetic engineering which I think could be applied more broadly. The first rule is if it isn't broken, don't fix it. As far as endothelial cell activation is concerned, yes, it's something to be concerned about, and it's a mechanism of graft injury that we've learned a great deal about, but we know that endothelial cell activation occurs in everyday life in all of us, and the problem may not be so much that endothelium gets activated, but that it doesn't revert back to the resting state or that for whatever reason the graft doesn't tolerate it. So my inclination is not to try and prevent or reverse something that we know to be normal physiologically. And again this goes back to the concept of accommodation. If the biological tissue or organ can sustain itself in the face of a given problem, my advice is that it's better to keep it the way it is than to try and prevent it because the second rule of genetic engineering is for every action there's an equal and opposite reaction. When you introduce a genetic change, say to prevent endothelial cell activation, the individual that's changed is going to change in more ways than you would predict, especially the knockout animals. If you look at the phenotypes of knockout animals that have been produced for research, very few of them were clearly predictable at the outset because their systems compensated, and other things increased in amounts et cetera. So I know this was a more complex answer than you had anticipated when you asked the question, but I'm not convinced that anything needs to be done about -- I hope nothing needs to be done about endothelial cell activation, but that we can look at those earlier steps.

DR. SYKES: Just one more question for Dr. Platt or Dr. Dalmasso. Do we know if antibody is required for accommodation?

DR. DALMASSO: I think the approach has always been to reduce the level of antibody as much as possible. In ABO allotransplantation the levels can go down very, very much, but sometimes there is trace amounts of antibody that one cannot detect by the current techniques that are used. So whether or not there is a very small amount of antibody that is required is unknown at the present time. I do believe though that trying to induce accommodation with antibodies is probably not an approach. I think that probably antibodies are not required, but I don't know what Jeff's opinion is about that.

DR. PLATT: Well, I would just cite your research, which seems to show that antibodies are not required. I mean you could induce accommodation in vitro using lectin as an example. Now, you might say, well, that's mimicking what an antibody does, but I disagree because our studies would suggest that lectin is acting differently. So I think your research suggests, hopefully for all of us, that it may be possible to induce accommodation without antibodies.

DR. DALMASSO: One of the complications of the field of accommodation and antibody is that when other investigators have used IgG antibodies to induce accommodation, in that case the antibodies were not necessarily against the antigens that have some bearing to transplantation immunity.

DR. SYKES: Dr. Collins, you had a question.

DR. COLLINS: Thanks, Dr. Sykes. I've got a question for Dr. Platt. I spent two years actually in Dr. Platt's laboratory, and that was a great experience. And as you can tell, he is certainly an expert in this field. Dr. Platt, putting on your clinician's hat, in allotransplantation there used to be a time when more immunosuppression was better. Patients have complications of immunosuppression. And in fact, in liver transplantation now a lot of patients are getting down to just FK506 and no other immunosuppression. Looking at the ways that primate recipients are modified such that hyperacute rejection is avoided, do you think all of those approaches are clinically feasible or is it too aggressive for patients?

DR. PLATT: Thanks, Brad. I learned a lot from you during those two years. I think it is relatively easy to prevent hyperacute rejection, so in my view this is not a problem. And there's a lot to be learned from it, but it isn't to be avoided. I think the essence of your question is really contained in the presentation that Dr. Zhong gave. What I drew from your presentation, aside from feasibility, was that many of the problems you are grappling with are unique to the baboon, how to optimize immunosuppression, et cetera, et cetera. And naturally you are then stuck relearning in an animal model system lessons that have already been learned with great difficulty, but nevertheless, learned in human systems, which is not to say not to use an animal model system. I think we need to do that, but I think at the same time some of the problems that we may be seeing might not be problems or might be handled differently in a human than they would be in a baboon or in a mouse.

DR. SYKES: Well, thank you very much, all of the speakers and all of the committee members, for their comments and questions. We are out of time, so I think we'll break now for lunch. Mary?

DR. GROESCH: Yes. I wanted to let you know that there's a restaurant downstairs, and there are seats set aside for the speakers and the members, the people at the table, and there's a buffet down there. You can get in and out quickly and be back here by 2: 00. There's plenty of room in the restaurant for anyone who's here at the meeting, and there's also restaurants very close by. But if we could please come back at 2:00 o'clock so that we don't short- change any of this afternoon's speakers because I think we have some really interesting talks there as well. Thank you.

(Lunch recess)

DR. SALOMON: Time to get started, so take your seat. Okay, well, everyone welcome back for the afternoon session, and I don't think I'm going to make any introductory comments. I think everybody has got the idea of the kinds of things that we're trying to get at, and so just to go right ahead, so we're going to try and stay on time, right? I mean everybody has got that, and we'll, as easy or as difficult as that may be. So the first person today is, this afternoon, is Gary Levy from the University of Toronto. He is going to talk about liver perfusion and bioartificial liver devices.

DR. LEVY: Well, thank you very much, Dan, and I again want to also echo the sentiments of the other

speakers, and thank you for asking us here.

So what I'm going to try and cover is I think this morning was basic science. What I'll try to do is put maybe a little bit of a spin, interject a little bit of basic science into where xeno is going, at least in terms of liver.

Now I want to introduce the topic of liver failure. Liver failure is a big problem, and it just depends, like this big mammal here, it depends which end of it you're looking at. If this thing was coming down this -- down the hall here, this would be a serious problem for all of us if you got in the way of the tusks, so just to give you an idea, there is a lot of liver disease out here, and there were some statements made, and I think our second speaker will talk about artificial devices for heart. If we were to list everybody who needed a liver transplant, we would probably start a pandemonium, so we're very conservative, at least, in many of the indications which we consider for liver failure.

Now, why do we need some sort of artificial device, or for that matter xenotransplantation? And the answer is because this, which is from the UNOS database, the number of patients being listed is rising astronomically to well over 20,000 patients now on the UNOS database when I spoke to them just a couple weeks ago. And in all countries, this is a very similar trait, success tends to bring people out of the cupboard.

The other thing you need to know we alluded to this morning is that whether or not we come up with a bioartificial device, or any technology towards the treatment of a patient, it depends on the nature of the disease. And liver disease has two spectrum. This is a young woman who unfortunately died, and presented to our institution with acute liver failure, where her liver was destroyed in a matter of at least what we think is weeks. And what she died of is brain swelling, and ultimately basically a cerebral death. And then on other hand, you have patients like this. This is a man who is only 38 years old, who had hemochromatosis, which is a genetic disorder of iron metabolism, and went on to develop hepatocellular carcinoma, and died of chronic liver disease. Now obviously if we're going to come up with a modality to treat patients, the treatment for the first patient who died of brain swelling isn't going to be the same type of treatment we're going to come up with for a gentleman who dies of chronic liver disease. This is outlined on this, just to give as an introduction. Chronic liver disease is a disease which is usually a long disease, in other words, patients have had it for many years. And people who die of chronic liver disease die of the ravages of that chronic liver disease. They don't die in general of liver failure. They die of the complications, and they bleed, and they often get an infection and die; whereas, people who die of acute liver failure die because the liver cells are gone. The intent -- The ability to maintain homeostasis is gone, and they develop brain swelling, and ultimately die a brain death. So if you get -- The interesting thing is if you get liver disease, though, in actual fact, people with chronic liver disease who develop these complications actually have a worse prognosis than the young woman I showed you who had -- who died of an acute disease. Not to say that they both don't do well, or either of them do well, but they tend -- The chronic patient needs a new -- an organ once he develops those complications.

The final comment I make before I go into the bioartificial devices is that liver transplant works, and that is the reason why there is so many people waiting, and this just shows you the results. And the results for even that young woman who presents in a coma bleeding from almost every port, we can anticipate that we can recover approximately 80 percent of those patients, if we can get the organs. But as all of you know, the reason you're sitting here is that the gap between supply and demand, both in the United States and worldwide, is widening as we speak, and the question is how do we meet that gap? Can we meet it with allotransplantation? Can we meet it with -- Do we need to consider other technologies?

Now just to show you the nature of the gap with liver disease, we are putting you, in the United States we're putting more patients, your doctors are putting more patients on the list than they're taking off the

list. So what that really means is that you're going to have patients who died -- who die rather than get the therapy which we all want them to get.

So we alluded to this morning that there are many different approaches to organ shortage, and to liver disease, one is allo, what I am going to refer to as these bioartificial organs. We alluded to a little bit about stem cells. I'll interject, because stem cells are becoming part of the bioexplosion, and then xeno.

Now just before I leave this, I know that when I sit on committees and I testify both in Canada and around the world people, say, "Well, why don't we increase allo or living related," just so you know, it is possible to take a piece of the liver, either the left or the right side of the liver, and put it into a person. The liver has a unique capability of regeneration. So we can cut out, and everybody who is sitting in this room, you could give half your livers, assuming they're normal, and we would end up with two full livers. That is the good news. This is an example. This is a son who gave half of his liver to his father, and both of them are doing very well. But as you well know, there are risks to doing liver -- living-related transplant, and Dr. Miller, who is in New York City, suffered that risk, and at the Mount Sinai Hospital he had a death of a donor, and that brings up ethical considerations of any technology.

So that is just a brief introduction into why we would consider liver support systems. And I want to just make a comment, liver support systems can have two outcomes. For the young woman with formal hepatic failure, the best outcome is that she walks away. In other words, we put her on some sort of bioartificial device, whatever that may be, and we keep her on it for a period of time, she recovers her liver regeneration, she walks away. For the patient with chronic liver disease, do not expect that kind of result. What we're talking about is a temporizing therapy until we can come up with some sort of transplant situation.

Now what are the liver support systems that are available to us? Well, first of all, we can do cross-circulation. This may sound a little barbaric, but you could bring an animal into a room such as a pig, hook in lines to that animal, and hook that patient up to that young woman, and see whether or not she survives. That has been done. That was done by George Abouna in about the 1950s and successfully done where patients actually walked away. You can do extracorporeal liver perfusion, which I'll go into, create a bioartificial liver, so the difference between this and this is we take a whole organ out of either a human or an animal, hook a patient up to it for a period of time, create a bioartificial liver device, whether that be cells, stem cells, whatever the case may be, and as was alluded to this morning, even consider hepatocyte transplantation, inject hepatocytes, whether they be Porcine, as Jeff Platt alluded to, or human hepatocytes. We actually did this many years ago when I was a medical resident many years ago. We actually hooked a patient up to a Porcine liver and wrote up the paper, and actually the patient walked out of the hospital. So this was a gentleman who had serious liver disease, and we took a Porcine liver, we didn't have this great device which is nothing more than an elaborate dialysis machine, and we perfused the liver using the human blood, collected the bile external, did not have the bile from the pig going through the patient, but basically used this to both clean the blood, as well as to replace factors which the liver didn't produce.

Now, the, as I said, the extracorporeal function could be looked at in the following way: It can function as a bridge to transplant, and those of you who are on the -- on the advisory committee, or who are involved in the trials that have been proposed and are ongoing in the United States and worldwide, this has become the major reason for extracorporeal perfusion. The next is to determine whether a patient will even benefit from liver transplant, so believe it or not, hook a patient up to see whether or not they could rally so they could become suitable, in other words, they may have crossed the line, they're too sick, put them on this to try to get them back to liver transplant, and so forth. And the question is to test whether poor quality human livers really are poor quality, and Ira Fox in Nebraska has used discarded human livers to show that these can function, at least in an extracorporeal situation. So as I mentioned, this is not

new technology. It was described by Otto in 1958, it was studied extensively in the '60s and '70s using both human and Porcine livers, George Abouna in Calgary, in Canada, hooked six patients up to pig livers, and in some cases he even brought the pigs into the patient's rooms, Eiseman also did it, and all of these individuals undertook extracorporeal liver perfusion, largely using pig organs. And in general, the conclusion from it was that it at least provided a temporary solution.

Ira Fox regenerated the whole field, he is at Nebraska, and in 1993 he reported successful application of extracorporeal perfusion using discarded, or non-usable human livers for the treatment of patients with fulminant hepatic failure. In general, this experience largely proved a bridge to transplantation, not a solution for the patient with liver failure.

Now, Dr. Chari, and we wrote the editorial on this at Duke University with the Duke group, treated a number of patients, this was the first series that was reported in the New England Journal in '94, treatment of a number of patients with hepatic failure with ex vivo pig liver perfusion followed by liver transplant. So what they did is very much the cartoon I showed you. They put a catheter into venous systems. They hooked the liver in circuit, oxygenated it, collected the bile, and had the patient hooked up for a period of time.

Now, I apologize for the quality of this picture. This is the liver during the perfusion. And I just want to tell you something can be too red, and I'll show you what I mean by that. But in actual fact, as we pointed out in our editorial, this was a pig liver which was undergoing, if not hyperacute rejection, certainly vascular rejection. It was mushy and soft, ah, associated with it. But when they looked at these patients, and the question was, was there a benefit, one can see that there was an improvement in performance of the patient with, in general, most of the patients had at least some improvement in their biochemistry, improvement in toxic compounds which exists in their blood, they were lowered in general, and even, as Dr. Robson pointed out, however, there were coagulation problems associated with this. And almost all of the patients who were hooked up developed DIC, which led us to report in the New England Journal that one has to be very careful with implementing this type of technology. One can do damage as well as good.

Now we started a whole research program with the University of Western Ontario looking at the role -- the potential role for the transgenic pig liver, and this would be it's either out in publication, or will be out in publication shortly, and this is one of the pig livers that is hooked actually to a baboon. And Bob and I spent long days and nights caring for animals while we hooked them to these devices. Now I just want to show you just briefly what we found from that type of trial. If one uses a non-transgenic pig with a normal baboon, remember these baboons did not have fulminant hepatic failure, so they have full complement levels, as Dr. Dalmasso pointed out. They have full plasma coagulation levels, one, which I'll show you in a moment, these livers undergo vascular rejection. However, interestingly, with the hDAF transgene, they were largely protected from that. So let's take a look at the liver histology of an extracorporeal perfusion apparatus, and here is the liver from -- It was hard to tell this was liver when we first looked at it. We originally thought this was spleen, because there is too much blood associated with this. And in actual fact the liver underwent hepatic necrosis. There is hemorrhage in the liver, and there is dense fibrin depositions, as many of the speakers have shown you. So using a normal non-transgenic pig in a patient who has full complement and full coagulation parameters can be dangerous. And Leonard Macalla, he worked with Nantes, he showed this by sewing a liver into a young woman, and when he gave her coagulation, in actual fact he produced this type of a picture in vivo. The hDAF in contrast actually perform very well. Histologically it looked normal. This may not look normal to you. It may look a little, for those of you who are aficionados, it may look like cirrhosis, but the pig liver actually looked cirrhotic, it's multilobulated, but the blood vessels are perfectly normal. So the hDAF protects against what Dr. Dalmasso and Dr. Platt talked about.

The next question is does the liver work? And the only way to answer that is by measuring the factors which Dr. Robson talked to you about today. This is a pig coagulation factor. We set this up in our laboratory. In actual fact, the pig liver produced very, very nice quantities of coagulation factors. So it worked, and generally it produced bile. The liver started to generate bile. So the animal was reasonably well-controlled, and homeostasis was kept. At the same time, we did not see massive liver necrosis. This is just a measure of liver necrosis. It's an enzyme that is released if you have serious liver damage. So we proved from these experiments that one at least in an animal model could undertake this.

So can you do it in humans? And the answer is yes, Marlon Levy, who is no relation to me, but he's at Baylor University with Goran Klintmalm, undertook, and we helped them a little bit with this study, they did some clinical work in hooking pig to human, or rather extracorporeal perfusion of two or three patients with fulminant hepatic failure who had these transgene inserts. And the answer is I won't show the data, these patients survived to go on to liver transplantation. Their biochemistry improved. Many of them woke up, and so therefore this was proven that one can use extracorporeal perfusion.

Have there been other forays into this? And the answer is yes. Omaha, Nebraska has now treated eight patients with extracorporeal perfusion. Most of these were with non-transgenic pig grafts, and as long as they didn't reconstitute the complement or coagulation, the patient seemed to do well. In the few where they did make the mistake of reconstituting, they saw vascular rejection. Duke has now done seven patients. I don't have the details on all of them. Two of them are alive. All of the patients, though, in general, have gone on to transplantation. So it has not served the purpose we wanted, which was to take patients with fulminant hepatic failure and restore them to normalcy.

So that brings us to the next device one could consider. This is not a computer, although it has computer parts in it. This is a bioartificial liver device, and we had this in our institution. There were two devices that were produced, one by Searcy's, one was produced by another company. The difference between them, they both had cartridges, you notice these cartridges in here, and so the blood enters through here, it is filtered through these devices, and basically there are columns of liver cells, either porcine or human, to try and both restore synthetic function and remove toxic substances from the liver. So there are a number of issues with bioartificial livers. First of all, where are the cells going to come from? Are we going to take them from pigs? Are we going to take them from humans? How do we prepare them? Should they be primary cells? Should they be secondary cells? How much cell mass do we need? 10 percent? 20 percent? A hundred percent? Should they be immune isolated to prevent some of the immunologic reactions that people have talked about? What is the best perfusion? Should there be whole blood? Should it be plasma? If we perfuse with whole blood, we invariably get activation of coagulation, as Dr. Robson pointed out, and we get DIC. And what is the best vascular access? I don't have time to go through all of this today.

You can either use cancer cells, transformed human liver cells, which are cancerous, that has been used, you can use immortalized liver cells which have been transformed with viruses to allow them to exist, or you could use stem cells. And in pigs, once again, the same sort of phenomenon, primary liver cells, immortalized or cryopreserved. The problem with, of course, transformed cells is, one, they may have viruses in them. Some of them may be tumorigenic. What happens if a little piece breaks off and lodges in the patient? What about immunosuppression post liver transplant? Do they still maintain differentiated function? So there is a series of questions, as one moves from primary liver cells to secondary liver cells as to function and obviously risk.

Just to give you an idea, this was a slide from a device we actually made to treat animals, and basically what we do, these are the cartridges. They have long filaments, and basically you grow the cells inside one of these little filaments, and you nourish them with growth factors and plasma, and then you can perfuse outside. There is a barrier not to allow plasma or blood to interact. And this is just an electron

micrograph to show you some liver cells inside one of these hollow filaments. So you basically inject these liver cells into these cartridges, and then perfuse them. But there are other strategies that people have tried, ah, and that is to actually try to grow, regenerate livers, taking both endothelial cell cultures, and then try to reproduce hepatocytes. And this type of work is ongoing by Dr. Nyberg reported as, and I'll show you some exciting work from G.A. Biconte (phonetic) in Boston who is trying to create a computerized model of a liver.

I've already alluded to the fact that one could either perfuse these with whole blood or plasmapheresis. The problem with whole blood is that you have to anticoagulate the patient. I don't have to tell you these are very desperately ill patients, and so there is a risk. And in one clinical trial done with an ELAD, an extracorporeal liver device, and with systemic anticoagulation. A number of patients bled into their brains because of the use of systemic anticoagulation.

In contrast, the device which Dr. Demetriou has been working on in collaboration with Searcy's does not require anticoagulation. It's a plasmapheresis reagent. It isolates the bioreactor from the patient. These are pig hepatocytes. The problem, of course, is you need a plasma separator, and it does limit the viability of these cells. They have to be replaced much more quickly than if they were perfused with blood.

So what are the results of clinical trials? I've already told you there are two major entities that are going on, one by Dr. Demetriou with Cedars-Sinai, which is a multicentered trial using cryopreserved Porcine hepatocytes attached to microcarriers in combination with plasmapheresis and charcoal. And Dr. Sussman's device, which is no longer in use, which use tumor cells, a primary -- a secondary human hepatoblastoma, this time using blood perfusion.

This is from Dr. Sussman's work, just to show you, that when one looked at actually a function, as measured by this parameter, galactose elimination, which is just an elimination of toxins, in a series of patients which he studied, he found these patients, their performance was markedly improved at the time they were hooked up to these columns. Furthermore, if one looked at their biochemistry and their physiology, their lactate production, you need a liver to get rid of lactate, that is a molecule that is produced, it is -- For those of you who run, and you get cramps, it is lactic acid. It is eliminated in the liver. And he showed that with this device, he was able to produce that.

Now Dr. Demetriou, I'm not going to show you his latest results. He'll be presenting that both at -- in Washington at the transplant meetings and also in -- at the ASLD. But is in his first experience, he treated a number of patients with fulminant hepatic failure, and he found that in general he was able to show that their brain swelling was markedly reduced when we hooked them up to the cartridge, and in this particular study, all of them went on to transplantation successfully. And they're all alive and well. And his conclusion was this was evidence that it worked.

Now in his latest study, when I talked to him just a few days ago, he said some of the patients now with fulminant hepatic failure have actually walked away from their disease with his device.

This is Dr. Vacanti. He loaned me a present, this particular picture. Jay is producing basically a three-dimensional liver using -- he is actually working with computer modeling and producing endothelial cells. He is making basically a scaffolding, on which he lays the endothelium which we heard about today, and then he puts hepatocytes along those chambers. And he is reconstructing a liver and using pressure transducer to control arterial and venous blood. And he hopes to be in clinical trials within 18 months.

Finally, I just want to leave you with the following: We now have, and we alluded to the fact that there

are stem cells, and this may prove to be a good source of cells. We know that even from the blood, we can find cells, this is from Dr. Demetriou who just published this, and he has shown that he can take stem cells from the blood, and they can differentiate into hepatocytes producing albumin, coagulation factors, and so therefore there may be the possibility of an endless supply of liver cells for bioartificial devices.

And just in final, the liver is not a dumb organ, and the issue is there are 20 thousand enzyme systems in the liver. So if one were going to predict which organ one would use for xeno, you wouldn't choose the liver, because you would predict that the 20 thousand enzyme systems wouldn't work. Well, I'm sorry to tell you, they do work, and here is an experiment that was done in Edmonton, Alberta by the group Loren Tyrrell and Norm Kneteman, who made a mouse with a transgene, and it's a scid mouse, and because of this transgene, it overexpresses a molecule which kills its own native liver cells. And what they then did was infuse human hepatocytes into that mouse, and interestingly, the mouse then repopulated its whole liver with human hepatocytes. So it really is a chimeric animal. It has no mouse proteins. It doesn't look like a human being. It looks like a mouse. But it does make mouse proteins. And for those of you who want to read this, it's a fascinating paper. What is interesting is that despite what Simon and I would have predicted, the coagulation works perfectly normally. The endothelial cell function is perfectly normal. The complement system is perfectly normal, this mouse, for all intents and purposes, is normal. So we may not have these physiologic impediments that we thought we would have prior to this type of experiment. And it may be possible for Porcine hepatocytes to do the function in humans. And this just shows those livers as they repopulate with human, they start out, this is the pig, these are the mouse hepatocytes. And by the end, they're almost totally human hepatocytes with just human proteins.

So I'm going to close by saying I think that the area of bioartificial for liver is blossoming. I think that, first of all, extracorporeal perfusion is still a possibility. We are waiting for the alpha-Gal knockout pig, or for that matter, even the hDAF proved to be successful. I think irrespective of that, the concept of building an artificial liver is becoming closer to a reality. Thank you very much.

DR. SALOMON: Thank you, Gary. That was outstanding. We can do a couple quick questions. The way -- We have the afternoon organized a little different than the morning, where it's kind of in theme, so there is the extracorporeal and bioartificial, so the next speaker will talk about bioartificial devices for heart, and then we'll have a discussion, so if we can -- if there is questions that, like, just really specific, otherwise what I'd like to do is just hold them, go on to the next speaker, and bring you both up, and then -- okay.

DR. GROESCH: They have to switch computers, so if there is a question, this is a good time.

DR. SYKES: I have one, Gary. A very interesting paper that you showed us on human hepatic function in the mouse. Did they look at cholesterol metabolism?

DR. LEVY: They haven't looked at all of that, Megan. I think they were surprised at the viability. First of all, this mouse is actually being used, as you may be aware, to study hepatitis C. So first of all what they've done is created a group of mice with human liver tissue, some are normal human liver, and some have hepatitis C in them, so they're actually using it to grow hepatitis C, and potentially to study its kinetics and all the rest. I don't know about cholesterol. They shared some of this data with me. We have some of these mice now, and we're just in the process of studying them for the potential of all their metabolic functions. But I don't know about cholesterol.

DR. SYKES: I see. Thanks.

DR. SALOMON: Okay. Thanks. Harold.

DR. VANDERPOOL: Dr. Levy, just one question. Were studies of -- of PERV done on these devices, and could you give us something about the results?

DR. LEVY: Yeah, sorry about that. Because of time, we obviously couldn't cover everything. The answer is yes. First of all, we have two patients who have been treated with these extracorporeal perfusion. There is one in Montreal as well, and they've been studied extensively. Our patients are now about three years out from exit, they have no evidence of PERV whatsoever. Interestingly, they were hooked up to the Porcine. They do -- They are chimeric, interesting, we wouldn't have thought they would be, because Dr. Demetriou assured me that the pig cells couldn't get into the patient because they're in these cartridges, but they do have pig sequences in them. So whether it's cells, it's hard to tell. It's a low number of copies. In the study by Marlon Levy, who again is not related to me, just a namesake, they have been following their patients, and there has been no PERV whatsoever. So there has been no evidence of infectious risk. And I think Dr. Chapman has been -- may have done some of the work with some of the patients in the United States.

DR. CHAPMAN: We have looked at, and while in the lab at CDC we looked at the Searcy patients, and that data was presented by Zorina Pitkin at the International Xenotransplantation Meeting last fall, and forgive me, I forget also at a liver meeting, and the paper is in preparation. But we've screened the patients through their phase two trials, and I forget exactly how many patients, I believe it's a couple of hundred, and found no evidence of infection.

It's maybe also worth mentioning that there are a couple papers in the literature, not looking at exposed patients, but looking at bioengineering on these, the contents of these devices, specifically showing that if you change the pore size of some of the components, how these cells, the pig cells, are inside of a component, and depending on the size of the pores in the component, you can get pores that are small enough where the device still works, but the PERV can't move out outside through those pores because it's too big. So there is actually some -- some preclinical studies, I guess, bioengineering studies like that out in the literature now as well.

DR. SALOMON: There is pretty good data now that PERV does not go through the current generation. But the other thing, just that you point out, Gary, is from my experience back in the old days with nephrology, we had -- There is a certain number of these fibers that break, you know, and that is what -- that is what limits reuse on dialyzers, so during the period of time where we were reusing dialyzers, that is a practice that kind of ended. But you could reuse them about five or six times, and then there would be a certain amount of breakage. I think you got to realize we were not surprised there were chimeric cells in them.

DR. LEVY: No, we don't reuse them, though. I think we learned, Dan, it's always good to be second on a situation like that, and so the whole -- the whole policy was not to use/reuse these devices.

DR. SALOMON: Okay, well, we'll have a chance to do more discussion of this, unless it's got to be right on this one?

MS. ENGSTROM: No, no, I can wait. Something Dr. Levy had said, I wanted to get some clarification.

DR. SALOMON: Why don't we do Dr. Michler's thing, and then come back to that. I'll start with you when we start. So the next speaker is Robert Michler. He is going to talk to us about current and future therapies in mechanical circulatory assistance. And maybe later we can get him to engage us also with -- He was one of the people that I think had very clear contributions to the debates that we had in the FDA Advisory Committees over should we go with hearts, should we go with children, and he was very articulate in that area as well. So we'll try and drag that into it during the discussion.

DR. MICHLER: Thank you, Dr. Salomon. Thank you very much, Dr. Vanderpool and Dr. Sykes, for inviting me to join you today. What I'd like to begin by discussing with regard to the current and future therapies in mechanical circulatory assistance for replacement of the heart is a simple fact that the heart has been a source of great interest in mythology, it's formed a great fascination for mankind, but one of the great -- greatest obstacles we think we face as human beings is the fact that we are grappling with a huge public health problem with heart failure. And I would like to compare what we are trying to achieve in the management of heart failure with a very simple metaphor, and that is the metaphor for the first descent on Mount Everest. Any mountain area expedition is an incredible challenge, and that challenge requires great preparation, and often the opportunity to look back and understand where we are. So if we begin by evaluating the challenge, and that is the failure of heart -- of the burden of heart failure, we begin by recognizing that heart disease is this nation's number one killer. In fact, it is the number one killer of most developing countries. It kills more people than the next seven leading causes of death added together. In this nation, there are about four to 500 thousand new cases each year in the U.S., about five million patients are affected. Two million of those patients are under the age of 65. Its cost is extraordinary. It's in fact the most common DRG in this country. It kills nearly 42 thousand people each year, affecting women more than men, and it is the only form of heart disease that is actually increasing in its -- in its incidence and prevalence.

If we look at the current therapy for heart failure and we compare medical therapy, we see that the one year survival with medical therapy in patients with advanced heart failure is about 50 percent. If we compare that to a patient who receives a transplant, we now have literally increased their survival 10-fold, so that their 50 percent point is now at a decade. And this is really quite extraordinary. When you think back to December 3rd, 1967, when Christian Barnard and colleagues performed that first orthotopic transplant procedure. I think little would they have realized that that extraordinary event has led to probably the Achilles heel of heart transplantation, and that is that it has become such an accepted therapy, that we are no longer able to apply it to even a small fraction of the population. In fact, I think it's safe to say that heart transplantation is epidemiologically inconsequential. It makes truly no difference, in the face of this extraordinary problem that Americans face day in and day out.

The numbers of transplants sadly are dropping worldwide, and in this country, the number has not increased much over a plateau of 2000 transplant procedures in a single year.

So that of the 30 thousand patients listed for cardiac transplantation worldwide, a third will be known to die. This is the reality. This cartoon art, once again, depicts the reality of the situation, and this sadly is the current era in which we face ourselves today, and sadly, more importantly, what our patients face day in and day out.

So what are the potential alternatives to conventional heart transplantation that might in fact have a more epidemiologic impact? Well, I think the topic of my discussion today is a mechanical assist device, but the topic overriding this session is really that of consideration of a biologic assist device, and that is the euphemism I like to use for what xenotransplantation is all about. The goals for either therapy, or both therapies are really the same. What we want to achieve is rehabilitation of our patients, we want to rehabilitate their end organs. We want to provide them with physical, emotional and psychological rehabilitation, improve their quality of life. We want to be sure that these devices either, one, has acceptable morbidity, that the devices themselves are reliable, and that these patients can in fact return to their families and to society.

Let's begin by discussing these alternatives. What is a mechanical assist device? One form of mechanical assistance is an acronym that you probably have heard many times, that is an LVAD, which is a left ventricular assist device. The other is the total artificial heart. You probably are all familiar with the total

artificial heart that Bobby Clark received, a complete explant of the heart, and a Jarvik 7 device was implanted in Barney Clark.

A left ventricular assist device, on the other hand, does not prior complete explant of the organ with the inflow to the device being attached to the apex of the left ventricle, and the outflow of the device being attached to the ascending aorta. So the heart becomes basically a low pressure system. This is the pumping device. There are many different models that I'll show you in a moment, and then there is some form of percutaneous drive line in certain systems that is a form of power, as well as control, to control a patient as much as a pacemaker would, as they increase their activity. The device itself increases their activity.

Let's begin by looking at what are the devices that are currently out there that have been FDA approved? These devices are all ventricular assist devices. There is no total artificial heart that has been approved by the FDA. These are the devices, and I would like to go through them with you in just a moment, but let's talk in general for a moment about what the pros and cons are for these mechanical devices.

An advantage is that the native heart is left in place so that it forms a backup in the event that the device has a catastrophic device malfunction. It's generally a technical straightforward implant. But I really want to focus on one very important issue, and that is that current devices are very large, and these devices exclude most children and many women. And who would have thought that when the NIH was funding this kind of research, and the government the NHLBI and others were funding this kind of research, they in their wildest dreams would have thought that they were imposing criteria on research laboratories that would actually create a device that was sexist. And I say that very seriously, because these devices are made basically for men, basically for people who are over the body size of about 125 or 130 pounds. And that simple decision tree was based on the fact that they imposed upon investigators the fact that these devices had to deliver a certain prerequisite cardiac output. And in so doing, it made the devices big, and in so doing, eliminated a huge population of patients with heart failure. And as I showed you at the beginning, 25 thousand women die in this country every year because of heart failure.

The challenges have been the challenges of thromboembolism, reliability and durability, as well as infection. Infection is in fact the Achilles heel of these current devices. Drive line infections, pocket infections, the device can itself become infected, and we have coined a term called device-related endocarditis.

Let's begin by looking at the Abiomed BVS 5000, which is approved as a bridge to recovery. This device actually is a fairly straightforward implant. It can provide function for both the left and right sides of the heart, it's a paracorporeal device, meaning that the tubes stick out of the body, and that the device, this is actually the device right here. It bumps in the two valves here. This is the device pumping for the left and right-sided system with the console, and what goes inside the patient are the inflow and outflow cannulas to either the left or the right side of the heart. Unfortunately, it's difficult for a patient to be mobilized with this kind of system.

The second system is the Thoratec, which is a bridge to recovery, as well as a bridge to transplant approval. Again, a straightforward implant requiring connections to the left and right side of the heart. This is also a paracorporeal system, meaning that the device sits outside of the body. These patients are able to be mobilized a little more effectively than the Abiomed BVS 5000 system. These patients do require anticoagulation. It is a pneumatic driven system, and some smaller patients can fit into this device because -- not because of the -- for two reasons: One, the cannulas inside of the heart have been made smaller. But secondly, it's a paracorporeal device, so even in a smaller patient, this device can sit outside of the body. And unlike some of the other devices I'm about to show you, which have to sit inside of the body cavity. This is the Thoratec device. It has the blood contact surface area is inside here. This is the

power line controller inflow/outflow cannulas.

The console itself is the size of a refrigerator, basically, and this is a fairly complex system to set up, and has to be mobilized with the patient. There is an FDA-approved device for in-hospital use, and there is a clinical trial going on, excuse me, for discharge to home in the U.S. as well as in Europe.

The Heartmate device, which is a pneumatic as well as a vented electric device, has been approved by the FDA for bridge to transplant. This is also the device that received recently a lot of attention in the REMATCH trial, which was a trial as a destination therapy. The device had demonstrated a doubling of survival of two years, a tripling of three years versus patients medically managed, but had as high as a 30 to 40 percent complication rate.

This is what the device looks like. This is the external pneumatic drive line, the outflow graft goes to the aorta, and the inflow cannula that gets inserted inside of the left ventricle. It has a pusher plate. This is a cinctured titanium surface and a polyurethane surface that is corrugated, it looks a little bit like the surface of an English muffin, and therefore forms its own endothelium. For decades people tried to find the smoothest alloy on the planet in order to put into these devices, but this kind of intuitive notion that you create a corrugated irregular surface, and then the blood components of the body would laminate it with a smooth surface in fact proved to be quite efficacious, and these patients don't require anticoagulation, but simply an aspirin a day.

Again, infection is an issue with both the vented electric and the pneumatic device. This is the electrical device with its battery size, or VCR sized batteries. Again, this is the vent to the electric device with transcutaneous coils. This is what it looks like on a model. We never have a patient in heart failure who really looks that good.

The other device is a Worldheart Novacor device that has been approved by -- for a bridge to transplant. A number of these companies have purchased other devices, so you'll start to see in my slides, the Heartmate, also called the Thoratec, which were once independent companies, Worldheart is a Canadian company out of Ottawa, which has the Ottawa heart that currently is in investigative trials, and they have purchased now the Novacor system. So it gets a little bit confusing. But the Novacor system has really been the most reliable. It has mechanical valves in it, so it's really quite durable, as opposed to the Heartmate device, which has biologic valves. This device unfortunately has had relatively high thromboembolic rate despite anticoagulation, and that has been a problem with this particular device.

All of these patients who are -- get these devices, excuse me, can be managed as outpatients. And this becomes a major issue for them. And it can be quite complicated. We have to educate the patient and family, we have to alert EMT personnel locally, we have to advise the utility company where they live that if the power goes off in their neighborhood, that it's very important that the power go on relatively soon. In addition, they have to live in close proximity to a device center. So I think it's fairly safe to say that current left ventricular assist devices have been mostly safe and effective. But can we do better? And let's look at some of the new devices and some of the clinical trials that are under way. We know the drawbacks are the large size, the thromboembolic rate, reliability and durability, biologic components, such as the biologic valves, power requirements, and the infection rate.

Let's look at -- at these devices at totally implantable, and there is a totally implantable left ventricular assist device, as well as totally implantable totally artificial heart, one of which you all recently read about is the Abiomed AbioCOR. So let's begin by looking at the Penn State Arrow heart, or the Lionheart. This device is the first totally implantable device of any kind to be used in the United States. It preceded the device that you heard about out of the University of Kentucky with Raymond Gray and his group. This device is designed as a destination therapy. It's currently in clinical trial. The first implant was is in

October of 1999. And it has transcutaneous energy source, a controller. This energy source is able to transmit energy outside of the body. There is no percutaneous leads. This is what the device looks like laid out on the operating room table, with the device here, with the controller, the internal coil, and a compliance chamber for venting air and -- and fluid.

This is what an x-ray looks like on a patient, and I -- I wish to thank the Hershey group, Dr. Ben Sun in particular for having loaned me this photograph. But this is what the device looks like. This is what it looks like in the -- in the abdomen, and clearly this requires a patient of a fairly large body size.

The next topic is the total artificial heart, Abiomed's AbioCOR. This is something that requires significant attention, as to why one would consider a total artificial heart as opposed to a left ventricular assist device.

We know that the advantages are that it can supply both right and left-sided circulatory support, valve pathology inside the native heart is inconsequential, the large size of the unit, however, limits the implant population. Estimates suggest that only -- The total artificial hearts would only really be necessary in about 15 to 20 percent of the population. So through all the excitement, a total artificial heart is likely to only serve a small segment of the entire population. This is what the device looks like. This is what it looks like with its battery pack transcutaneous coil, controller. To date, the FDA first of all permitted this to be investigated in the U.S. in January of 2001. There have been six implants, with three deaths, one of which was a very well covered by the media, and I'm sure you all saw. That patient unfortunately died of a stroke, which is one of, again, the issues. The FDA has looked at the data, and has approved the five sites to put in an additional six devices before re-review.

It's a fully implantable device. It is a big device, and again has the technology for transcutaneous energy transfer, which is a significant issue.

Ah, I -- This, I want to show you simply because I think that the companies themselves can often be extremely misleading in terms of what the device can do. This is obviously someone on the golf course. He's got his golf glove on. He is a very slender, probably extremely attractive individual who has got a device that probably wouldn't fit in him.

The Abiomed Penn State AbioCOR 2 device is a device that was designed by Penn State, purchased by Abiomed for strategic reasons, and probably the strategy behind it is the fact that this device is actually smaller. Now Penn State did something very smart, when they were told by the NHLBI and the other funding agencies that they had to achieve a certain cardiac output, they said, "Fine, we'll design a device that can do that, but we'll also design a smaller device in the hope that that device as well would one day become clinically applicable," and that device is probably going to be the device that we see in many trials and see application of.

This device is currently in -- being studied for durability and in animal trials. The Cardiorest device should be mentioned as a total heart artificial heart mostly for historic reasons. This is actually the original Jarvik 7 total artificial heart, and no one wants to finance it, so it probably will not continue in clinical trials.

So what are second generation devices out there that are potentially available to us that might try and deal with some of these issues that we've talked about? I want to talk to you about two of these, one is the centrifugal flow pump, the AB180, a device we had experience with at Ohio State University. Also some axial flow pumps, the Jarvik 2000, which I had experience with in the laboratory doing some of the original work when I was at Columbia, New York, and Mike DeBakey's device, and the Heartmate II device.

The most interesting thing about these devices is that they try and answer a fundamental question, and that is whether unloading of the ventricle is really necessary. And this is from our laboratory. It shows a heart in an animal that has been induced into heart failure. This is the left ventricle. It's dilated, poorly contractile. This is the right ventricle. Now we're going to turn on the device here in a moment, and we're going to see the left ventricle shrink, the right ventricle become extremely large, and it's important to recognize that any muscle must in our belief, must be under certain loading conditions. See how that heart and septum shifts over here? It almost obliterates the cavity. The right ventricle dilates quite significantly, and it's probable that if one were to institute some kind of therapy during partial unloading of the heart, say for example, at this point here, where the heart is seeing some volume, is undertaking some stress, that inserting autologous myocytes, which we are doing a clinical FDA trial on now in humans, potentially even stem cells or other therapies. These hearts may be in fact coaxed into some type of -- of improvement and recovery.

So the issues with the newer devices are how to create smaller devices, make them easy to insert, lubrication of bearings, and what are the risks of aortic insufficiency if the device fails?

The AB 180 heart, as I mentioned before, this is a centrifugal pump. We use this as a bridge to transplantation, saved a patient's life with it, and also this has a unique characteristic in that it can be inserted percutaneously, and there is currently an FDA clinical trial to use this. I personally have had experience with the percutaneous device in that one of our cardiologists implanted it in a patient with cardiogenic shock who had severe mitral insufficiency. Two days later we took the patient to the operating room, explanted the device, repaired the mitral valve, and today, six months later, the patient is alive. Axial flow devices, on the other hand, are impeller pumps, like little submarines. The Jarvik 2000, the DeBakey pump and TCI Heartmate pump are all variations on this. They are smaller than a Novacor device, they are mechanically simple. They do create heat energy, and therefore require lubrication. The design is to have a longer battery life. The question is can they be made to have fewer blood clots? And that is an important issue. This is what the device looks like on cross-section. It has an impeller pump, a motor that drives the blood from one end to the other. So far, to date, this was the first device in clinical trials. Over a hundred patients have been implanted in Europe. There is no controller, so you have to dial this up if you want to exercise. Sadly, there has been a very high thrombotic rate. Some patients have been required to receive thrombolytics to unstick this device. And this has resulted in some deaths.

The Jarvik 2000 is a similar device that -- that has had eight implants worldwide. There is no inflow conduits, so the question is whether there would be fewer clots. But there is some question about whether near the aortic valve and the coronary arteries there can be stasis.

The Heartmate II device has had five to six implants in Europe, no U.S. trial yet, it is a small device compared to the standard Heartmate that I showed you before. So if you think of where we stand today in this field the mechanical circulatory assistance, I think it's fair to say that there is an incredible base camp of challenging and sophisticated research. And I would like to share with you really what is the third generation kind of pump which is extraordinarily exciting in the form of a magnetically suspended centrifugal pump in which the rotating component floats in a magnetic field while rotating at very high speed. This eliminates shaft and bearings, there is no bearing load, there is no heat generation, no need for lubrication, and presumably decreases the risk of hemolysis and no anticoagulation because of its design. There are three types out there: The Golding, the Kryton (phonetic), and the Heartmate III. This is the size of an apricot, and could be a very, very unique design for these devices. One of the things we have been challenged with is to create an animal model of heart failure, and that animal model of heart failure we think will bear fruit in being able to determine time dependent markers of heart failure, novel medical strategy, a new generation of left ventricular assist devices, and the ability to trial these.

Other possibilities are volume reduction surgery, myocyte or stem cell transfer experiments. So the real question is are we even close to the management of heart failure being at all approaching a point where this can be epidemiologically consequential? I think the straightforward answer is that at this point in time, we are not. These devices do provide an effective therapy, but they're limited by size constraints, design issues, infection, thrombosis, and I think we will continue to see a general application of these devices, a growing ability to treat patients with them, but we must remember that many of these devices to date have not intended -- been intended to treat the pediatric or female population, remembering that heart transplantation plays no favorites. It is as good for a pediatric patient as it is for a woman, as it is for a man, adult or child.

In my opinion, future treatment algorithms must apply the best available therapy to the patient's clinical condition. That might mean a human heart, it might mean a mechanical device, or it might mean a biologic device in the form of a xenograft.

And if you will permit me to close with one point of levity, I'd like to share with you what one day might be an alternative source of thoracic organs for our patients in need of heart and lung transplantation.

Thank you very much.

DR. SALOMON: Thank you, Bob. That was great. I think probably the way we ought to do this is just to have you sit back down, and we'll just kind of begin a discussion on the sort of where we're at, contrasting the extracorporeal and bioartificial technologies, obviously, with xenotransplantation.

I promised Ms. Engstrom that you would get to lead off, and --

MS. ENGSTROM: Actually I have three questions, one for Dr. Levy and two for Dr. Michler. When you showed your slide, which I think was cross-section of bioartificial liver, and you were showing either the cartridge or the fiber in which you filled up with liver cells, you used a term, the phrase "human and pig liver cells," and my question was really a point of clarification, did you mean in the same device you had a mixture, a combination of both type of cells? Or did you mean in different machines you had one that was dedicated to human liver cells and one to pig cells? Could you clarify that?

DR. LEVY: So the answer is, I apologize for the confusion, no. In general, to my understanding, there are no hybrid systems where people have used pig cells to augment human source. In general, the bioartificial devices are the pig cells in total, along that cartridge. That could be endothelial cells plus other cells in with hepatocytes, the parenchymal cells, or they would be human, either primary or secondary cells, but not together.

MS. ENGSTROM: Okay. Thank you. For Dr. Michler, I have two questions. One, you showed a slide in which you listed the major drawbacks to the devices, and you -- and it started off, I think the first one was the size, and ended up with infection as the last one. I wondered if you had listed the -- in some sort of rank order, signifying that the first item on the slide was really the biggest impediment, and that infection was the least significant of the drawbacks. Did you imply any ranking by the way you listed them? And if there is a ranking, could you tell us what you perceive, or what the transplantation community working from the angle of the heart transplants, considers to be the single biggest impediment?

DR. MICHLER: Well, there was no intention to rank these, and I think it's impossible to rank them, quite frankly. These are huge issues. The -- I think probably if I were -- if you were to hold me down and ask me what the biggest problem is, I would say the epidemiologic impact. These devices should be designed to provide a source of therapy for all patients, not a select group of patients. But each of those

issues is a significant issue, and in my opinion, shouldn't carry any implicit weight, or else one would focus attention on one as being more important than the other. They're all important, and must be dealt with.

MS. ENGSTROM: My second question was I didn't quite understand your description of the magnetically suspended device. I wonder if you could take a few minutes to sort of describe that to me.

DR. MICHLER: The -- These are devices in which a magnet is part of a device, and rotates around the device so that it essentially creates a magnetic field, and that magnetic field pulls the blood in one direction to the other.

MS. ENGSTROM: Okay.

DR. MICHLER: The beauty of that, and I -- Excuse me for not making it clear. The beauty of that is that there isn't something in contact with the blood producing trauma on the blood. And that those moving parts, that traumatic interface with any liquid that causes some -- a liquid to move through a tube, that -- that energy transfer to a liquid medium can cause damage to the blood cells, can activate and does activate complement, activates platelets in a different biologic environment, but does all of the above, activates fibrinogen, and can lead to clotting.

DR. SALOMON: So the questions I have that I'd like to have you guys both respond to, and that is starting, Gary, with you, if you put pig hepatocytes in one of these devices, or human Hep 2 cells, how close do you think right now we are to replacing liver function? I mean you were a little vague about the fact that did these last for hours and hours? Can you go for 20 days like with some of these, you know where I'm going with this, right?

DR. LEVY: Uh-huh.

DR. SALOMON: Then I'd like to have Bob respond in the same way, and that is how close to perfection can we get with an artificial device for cardiac function?

DR. LEVY: These are first generation devices. I don't want to make an analogy of a Ford to a Cadillac because some of you may think a Ford is better than a Cadillac. But these are first generation devices, and so I think where they are with isolated cells, what we have learned is that primary cells are better than secondary cells. So coming back to your comment of Hep G2, so for the panel, Hep G2, or hepatoma cell line, it's a derived cell line. It's a secular, which means it's undergone a transformation from what is inside you. It's D differentiated, so it's not producing the same materials, or removing at the same rate the poisons that are within your body now. So as first generation devices, the experience suggests that they, that the pig hepatocytes or human hepatocytes primary lines, which can be made, last for about 12 hours. And then the cartridge has to be replaced. And you get signs that the cartridge, and unfortunately with time you can't always go through, you get signs that the cartridge needs to be replaced from the studies that Dr. Robson alluded to, and what I showed, you can measure Factor 7, which is a clotting protein, and as it starts to deteriorate, it is a protein that has a very short half life, and is a very sensitive marker for function. It's a very -- You can't use albumin, because albumin is made to the bitter end. You've heard albumin with many presentations.

The second aspect, Dan, is that we're learning that isolated cells don't like to be alone. It's like any of us if we were stuck alone on Mars, we'd probably just die of loneliness, if nothing else. So we like to be in company with other people. Cells are like that, too. And so we're learning that there are other matrix cells, endothelial cells. There is a thing called stellate cells, and we're starting to develop what is called tertiary structures. So we're finding that not only do the cells perform better, they survive longer. So the

work with G.A. Biconte, and work that is in our lab, is showing that those types of devices may be extended for 24 to 36 hours, and may be even longer. As we go to a more complex matrix, they're harder to make, but once you make them, they have a greater longevity, and they function better.

DR. MICHLER: I think that is a terrific question to compare mechanical devices and xenotransplantation because in effect this is where we'd like to be with xenotransplantation. We have a device, a mechanical assist device -- devices that are saving lives. We know they do. We know that in an extraordinary landmark study, the REMATCH trial, that one of those devices, which is not even a current device, but the -- the -- the device that is now almost 15 years old, saved lives, doubled survival compared to medical therapy. So device technology has come a tremendous way to the point where we're no longer just investigating these devices in animals, but we're applying them to humans, and we're seeing successes. We still see patients die, and sadly, none of us like to see that, but we keep thinking what could be the next device that would be a better device for these patients? And so, I think, it's -- it's very plain to see that we are far from perfect. We are making significant headway with these second generation devices. The concept that we might not need an enormous device in a patient, the concept that some of these devices can be fully implanted, and therefore reduce the risk of infection, is good. But I remain a proponent of the fact that I think biology is in my opinion, better than mechanics. And if one could develop a biologic assist device that is totally implantable, that deals in the same manner with the issues that we deal every single day with in terms of our allotransplanted patients, mediastinal issues, you know, rejection issues, if we can convert a biologic assist device into a clinically applicable therapy, in my opinion, that is an extraordinary advance.

DR. SALOMON: Let me just follow on that. Is there a -- In one argument that we've had at the FDA Advisory Committees is this sort of ethical principle of using these as a bridge because of the restricted number of organs available. So one of the things I thought was really interesting is the premise that you put out, that if we actually use these and thought of them as assist devices, instead of as heart transplant surrogates, that its impact could potentially even be greater than simply being a heart transplant surrogate. Because that would allow the normal heart to regenerate. So how long -- two questions. How long would a heart have -- a pig heart have to survive in someone to begin to be useful to you as a cardiac surgeon in bridging someone, let's say, with a flail (phonetic) mitral leaflet, or after an acute myocardial infarction that might recover. And, secondly, is there ways to put these in without having to remove the native heart?

DR. MICHLER: Complex set of questions, and I'll try and address it as best I can. First of all, from a technical aspect, yes, the -- The first implants of human hearts in South Africa and in the United States often were used as, and I almost smiled to say it, but they were called piggyback hearts. And these hearts were placed in -- in -- connected onto the current existing human heart in a way that would function essentially as a booster for the -- for the native heart. So from a technical perspective, yes, it can be done. I think most heart surgeons would want to basically retool them themselves and practice that operation a few times before doing it.

The concept of whether a bridging -- excuse me, bridging as is a good one is a -- is a totally separate and more complex issue. Again, it is epidemiologically of no consequence, but there is much that can be learned, in my opinion, from using these biologic assist devices in that capacity. First of all, the native heart remains in place, and if there was an acute failure of the biologic assist device, it would provide the patient hopefully with some time so that they could get back to their native hospital.

The biologic assist device would function, and -- as an immunologic stimulant for the body, and there is a tremendous amount that one can learn, many of the -- many of the research and investigating works we have done in our laboratory over the years have in fact been heterotopic transplants by both hearts and kidneys in which the organ functions as an immunologic stimulant, and is not required to support the

circulation.

DR. LEVY: I think, Dan, from a standpoint of the liver, I tried to point out to the panel that if these devices, these assist devices or biological devices are going to have an impact of reducing the impact of disease on the need for transplantation in liver, it would be on the patient with acute liver failure. And that is where the effort on your question has to go. And those trials need to be conducted properly, and that was one of the concerns I had with the Searcy trial, because a lot of the patients were chronic. So they used them as a bridge. But coming back to the acute patient, yes. I think the devices have the potential maybe alone, or in combination with stem cell technology in the long-term. And we're not -- I'm not going to put a time frame on anything with science, but in the long-term, there is the great potential for regeneration. For that type of patient, this could be, for lack of a better term, a cure.

For the patient with chronic disease in liver, this technology will only be a bridge to another therapy, because once the patient declares with chronic liver disease the failure entity, they need some replacement. Now, that doesn't -- We all can, in the short-term, we may be looking at transplants, so this is going to put greater strain. But I did point out that might be living related transplantation, which today you wouldn't even consider in someone who is so desperately ill. Or it might mean xeno, if that were to become a reality, or it might mean some other formal therapy yet to be defined. But it would mean they would need some further therapy.

DR. SALOMON: That is excellent. Now we're close -- We are starting to go over time, so given the fact that I, you know, we probably should stay on time, Harold, certainly as chair you trump anything that I say, so you can go ahead.

DR. VANDERPOOL: I just have a quick question for Dr. Michler, and I wouldn't take the time if I didn't think it was important, Dan. All these mechanical assist devices you've -- you've outlined for us, are they able to be supple? Can you make a -- a mechanical heart supple enough to allow you to go to sleep with a normal resting heart? And for the heart to speed up and not make you dizzy when you decide to walk fast, or take a flight of stairs? I mean how -- You talked about the important factor of quality of life, and our Informed Consent Committee has talked about how important that is also. What quality of life could you foresee that a strictly mechanical heart could be able to give?

DR. MICHLER: I think a very good quality of life. The devices, the controller on the device will in fact sense blood volume, and so the device itself can increase its cardiac output. Not all of the devices have a controller. The -- Those second generation devices that I showed you, the Jarvik 2000, the DeBakey pump, they're looking at controllers, and don't have them, but could be made to have them. Again, additional hardware for the device. But that is key. Your point is well taken. With -- With respect to quality of life, these devices, to be clinically applicable and destination therapies must be able to have patients return to full activity. I will tell you a couple of anecdotes. I remember a young lady who we put one of these devices in just managed to squeeze the device in, that Heartmate II, the -- the one that was involved in the REMATCH trial. She was a cheerleader and decided that with the device, she would go back to trying to be a cheerleader, despite our concerns of doing her that kind of activity. That gets into a whole other issue that patients may not be their own best caretakers when it comes to taking good care of a device that they have in, despite your best design. Some device failures are simply the result of patients not taking good care of their device, much as you can't get them to stop smoking, or take concern for their diabetes or hypertension or high cholesterol.

DR. SYKES: Robert, I'm going to ask you the question that we, in the field of xenotransplantation always get asked, how many years do you think we are away from having total artificial heart that can -- that is an end treatment that can support life long-term?

DR. MICHLER: Having been asked that question about xeno several times myself, I can feel your pain. The -- the -- The data shows that currently that one device that has been looked at as a destination therapy, that Heartmate device, will probably be approved by the FDA within the next several months as a destination therapy. So it is upon us. It is now. Is it the best device? No. But it is a device that is likely to save many, many lives. It is a device that still has many quality issues, design issues, safety issues. But I think when compared, and this is very, very important, and I know we have talked about it at these meetings before, we are not comparing these devices to normal patients. We are comparing these devices, whether they be a mechanical or a biologic device, to the burden of liver failure, kidney failure, heart failure, whose patient's quality of lives are terrible, whose likelihood for survival is limited, and as I showed you, 50 percent at one year, roughly, for patients with end stage heart failure. So if we compare it to the appropriate population of patients, I think that we're really within less than a decade, and probably within five years for mechanical devices.

DR. SALOMON: Thanks, Bob. I think we're going to have to go on, and we'll have time to come back. Otherwise, everyone's going to yell at me for going overtime, and they'll forget that I was a nice guy and let you talk. We're changing strategies a little bit here, and the next three talks are on transgenic engineering and cloning of potential source animals. This has obviously been a theme that repeatedly people have returned to since early this morning where in a sense almost each of the speakers this morning were identifying potential targets for transgenic engineering. So I think where we're going to try and go this afternoon is exactly what can you do? I mean how do you transgenically engineer pigs? What's the future? Where is it all going? So the first talk is from Randall Prather, University of Missouri, Columbia.

Agenda Item: Transgenic Pigs for Biomedicine and Agriculture

DR. PRATHER: Thank you very much. It's always a joy to talk about one's research and some of the successes that we've had. You see my title includes agriculture. I'm in a college of agricultural, and I see the technology that we've developed here as having application not only for xenotransplantation, as you'll hear about later, but also agriculture, biomedical research. Anybody that uses pigs potentially has application for this technology. Justification? Well, the procedures for making transgenic animals other than mice have really been inadequate. We've heard a lot today about galactosyltransferase. There's other genes that may be interesting to modify such as myostatin. I don't know if anybody's familiar with double muscle cattle, but they look like Olympic body builders on steroids. That's due to a natural mutation in the gene called myostatin. It may be interesting to knock that gene out in pigs and create a knockout and see if they have additional muscle growth. So there's tremendous opportunities for impacting reproductive efficiency and genetic progress.

For creating transgenic animals, in the past we've relied extensively on pronuclear injection. The pronuclear injection procedure is basically you take your piece of DNA, you suspend it in a buffer, and you microinject it into the nucleus of a one-cell-stage embryo. The efficiencies are low. They're even lower in domestic animals than they are in, for example, mice. You can improve efficiencies a little bit by using something like retroviral transduction. That can create some other problems. But for domestic animals these low efficiencies result in really humongous costs. The per diem on a pig is a little bit more than a mouse, and when you figure the mouse has a gestation of 21 days and a puberty of 21 days in the pig, it's more like six months to puberty and four months gestation. You have to keep these animals around a long time. But also interestingly, at least with the pronuclear injection, a third of the founder animals are mosaic. What does that mean? Well, when you inject your piece of DNA at the one-cell stage, sometimes it doesn't integrate into a chromosome until the embryo divides to the two-cell stage, and then it only integrates in one of the two cells. You end up with an embryo that's half transgenic, half not. If that transgenic cell doesn't give rise to other eggs, well, then you kind of have a dead-end animal.

You can't make any more transgenic animals from it.

But the most important consideration with these technologies is there's really no control over the insert. You don't know how many copies of the gene go in. You don't have any control over where on the chromosome, much less which chromosome, the gene goes into, so it creates some real problems or potential problems. For making transgenic pigs there's been published methods using pronuclear injection, sperm-mediated transfection -- I didn't talk about that -- oocyte transduction, but the common theme of all of these is that there's no control over the insert. You mouse guys that are out there are going oh, yeah, this is old technology for us because we've been able to use embryonic stem cells for quite a while and make chimeras and produce animals with specific genetic modifications. In pigs there aren't any. We don't have that option. And so the alternative is to use nuclear transfer using a transgenic cell. This will permit a specific genetic modification prior to creating the animal.

Let me give you a little history. Nuclear transfer was really first proposed back in 1938 by Hans Spemann. Hans was really interested in nuclear equivalence. In other words, as an embryo develops and differentiates, some cells go to liver, and some cells go to bone, but do they inherit unequal -- and I'll quote -- nucleoplasm? He was under the impression that there was different nuclear inheritance between the cells, and so that caused certain cells to differentiate irreversibly into different cell types. And he proposed -- and again I quote -- a somewhat fantastical experiment whereby he would take nuclei from progressively differentiated stages of embryos, transfer those nuclei back to an unfertilized egg, and as soon as you got to the point where that nucleus could no longer recapitulate development, you reached the point where there was nuclear inequivalence or where some of the nucleoplasm, as he would say, was lost. Unfortunately the technical aspects precluded him from doing those experiments, and it wasn't until 1952 that Briggs and King actually were able to perform the experiments on some rather large eggs, and this was in frogs.

Due to the small size of the mammalian embryo, it's technically very difficult to do some of these same experiments, so it wasn't until 1986 when Steen Willadsen took some cells from an early-stage sheep embryo, 8 to 16 cell stage, and transferred those nuclei back to an unfertilized egg and was able to create sheep. We did the same thing and published a paper in 1987. And then in '89 we did the same thing in cattle and then in pigs in '89. The next significant progress was made in 1995 when Sims and First published that they could take a cell line and do nuclear transfer and be able to create a calf. Well, us cloners were kind of ignored by everybody in the world until Dolly came along in 1997. Dolly made a big splash, and I'll admit that Dolly was important, but there was another sheep born that year that was also very important, and that sheep was Polly. Polly was the result of taking a cell in vitro, genetically modifying that cell, and then doing the nuclear transfer and creating a sheep that was transgenic. In 2000 the first report of actually doing a specific genetic modification, in this case a knockin, was reported by McCreath, et al. Also in 2000 adult pig cells were used so that now we could use cell lines from pigs to be able to do nuclear transfer. And this year we reported a specific genetic modification, a knockout.

What are some of the candidates in biomedicine? Probably the application of these technologies is going to be first in biomedicine because there's the potential of a higher profit margin than in agriculture. And we have the example here listed on top, xenotransplantation. I might mention that pigs are used in other areas of research, and one example exists with the potential of using pigs in cystic fibrosis research. It's due to a single gene mutation. Potentially we could introduce that single gene mutation into pigs and create a pig model of a human disease, cystic fibrosis, so that we could have real guinea pigs to test treatments on rather than humans.

A lot of applications to agriculture I briefly alluded to are myostatin gene. I don't want to go into any details here. Just that there are a lot of potential applications in making genetic modifications for production agriculture.

How does the procedure work? Well, in the top middle we have our unfertilized egg arrested at metaphase 2 of meiosis. You insert a micro pipette, you aspirate up the first polar body in the metaphase chromosomes in a process called enucleation, although it's not really a nucleus, so it's kind of a misnomer, but that's what we call it. I don't think anybody wants to say euchromatization. In the upper left-hand panel we have our donor cell line. We can transfect or transduce. You select those cells. You may want to synchronize them. And then you do nuclear transfer. And basically one aspect of it is that you put the cell in the perivitelline space. I'll try to get the pointer up here. I'll point at it. You put the donor cell in the perivitelline space, and you can fuse the two cells together. Alternatively, you can a microinject the nucleus directly into the cytoplasm of the oocyte.

Now, for an example, especially for you nonscientists that are in the audience. You might think of the differentiation process as in dominoes falling over. Fertilization knocks the first domino over. Cleavage to the 2-cell stage, 4-cell stage, 8-cell, 16. When you get out to what's called the blastocyst, which we have down here at the bottom left panel, you have two cell types, the inner cell mass, and then you also have the outer layer of the trophectoderm. The inner cell mass differentiates different than the outer layer of the trophectoderm. The outer layer of the trophectoderm goes to placenta. Inner cell mass goes to the fetus proper. You might think of this as now you have two pathways of dominoes falling over. As that embryo continues to differentiate and develop and more tissues specialize, you get more and more pathways of these dominoes that are falling over. And it might even be that each represent a different repertoire of RNAs that are produced by those cells. Well, really what you're asking to happen when you do the nuclear transfer is going down to the end of one of these lines of dominoes and stand it back up, reprogram that nucleus, and here we show that it swells in diameter, changes in structure, you get a change in function. The pronucleus or the transferred nucleus is reprogrammed so that it starts over in its developmental pathway. This is what it actually looks like. On the left are some fetal cells. In the middle is an egg that we've removed the chromosomes from. We've transferred in our donor cell that's getting ready to actually fuse the two cells together to transfer the nucleus, and on the right-hand side is a potential litter of cloned piglets.

For my first experiment that I'd like to describe, we took some fibroblasts derived from two-day 35 fetuses, passaged them seven times. One of them we transduced with a reporter gene, the enhanced green fluorescent protein, and we selected it for 13 days in media containing an antibiotic so that you should kill off all the nontransgenic cells. In this study we did not clonally select the cells, so each of the animals that we potentially would produce would have different sites of integration. Another cell line, FF4, was not transduced, so it was not transgenic, and we used donor sites from prepubertal gilts that were matured in defined conditions in vitro.

Here we have the results of three embryo transfers, Y60, 047 and 0147. Into Y60 we transferred 51 nuclear transfer embryos from FF1 and 30 from FF4 to give a total of 81 embryos transferred to this single surrogate. She cycled back on day 26. The normal estrus cycle in a pig is about day 21, so it appears that it was extended. We really don't have much evidence for it in terms of any ultrasound. Number 47, we transferred 61 from FF1 and 36 from FF4, for a total of 97, and she cycled back on day 41. The bottom surrogate received 79 from FF1 and 51 from FF4, for a total of 130. We delivered five piglets from this procedure, and they were delivered almost exactly a year ago. We did microsatellite analysis, and I won't go into the details on that, but that confirmed that all five of these were from the FF1 cell line. When we then went in to do the amplification of the enhanced green fluorescent protein gene, what we found is that number 4 didn't have the insert, and so we didn't select hard enough, and some of those cells slipped through the selection process. The birth weights in these pigs ranged from 845 grams to 1,740 grams. You would think that well, they're clones, they ought to be the same, but each clone is not phenotypically a carbon copy of the original. Here's four of them at day 40. You can see that one's a little bit shorter in length and in height. So there's some epigenetics that's involved in determining

phenotype.

In another study we used some ear-derived fibroblast cells. This animal, number 4022, was created by transducing an unfertilized oocyte, then fertilizing it, culturing it to the blastocyst stage, and doing embryo transfer. When this animal was born, at four days of age we isolated a cell line from it, we digested the isolated fibroblasts and used these for nuclear transfer. We were able to produce four piglets from that last August. And you can see that two of the piglets are much smaller than the other two even though they are genetically identical.

This is a list of our first ten piglets that we had. Number 1, initial results, normal and healthy. Number 2 has a flexor tendon contracture. I'll go into more detail about the contractures later, so I'm not going to say anything else about those. This animal died at day 35 of congestive heart failure. Number 3 was normal. He was euthanized at day 116 due to complications from a bacterial infection. Number 4 and 5 were normal and healthy. Number 6 appeared normal when it was born, however, it developed chronic diarrhea. It had a decreased growth rate. We talked about creatinine. Its creatinine level sky rocketed, and we determined that it had low vitamin E levels even though there was vitamin E in the diet. We then supplemented with additional oral vitamin E and injectable vitamin E, but the animal ended up dying at about 130 days of age. 1 through 6 are from the same base cell line that I talked about. 7, 8, 9 and 10 are from this 4022, so they should all be genetically identical. Number 7 had polydactyly, an extra toe. It developed a carpal valgus limb deformity about ten days of age. It was temporarily corrected, but then she just started putting too much weight on it, and we went ahead and euthanized her at day 165. Number 8 had a flexor tendon contracture. This was one of those very small piglets. Our housing facility -- and you learn things by doing them wrong I guess -- the flooring where this animal was kept had the normal flooring for baby pigs. Well, this pig was very small at birth, and its feet actually could slip through some of the flooring, and it ended up getting an infection around its hoof and died at three days of age. Number 9, again another small piglet, died at seven days of age from congestive heart failure. And number 10 fractured its distal P1 at six days of age. It healed normally. It's now healthy and starting to cycle.

So we have the technology to make the transgenic animals. The next question is, can we make a specific modification? And I'm not going to go into very much detail about this. Dr. Greenstein is going to cover some of the molecular aspects of what we did in the next talk, so I'll briefly talk about in a general sense what we did. Here we have DNA from a normal pig. They have genes that encode for galactosyltransferase and put the cell surface molecule, in this case galactosyltransferase, on the surface of the cell. Ideally what we'd want to do is go in and make a genetic modification so that that molecule is no longer on the surface of the cell so that those natural antibodies would not recognize it.

To do this experiment, again day 35 fetal fibroblasts, they were passaged a couple of times, plated in 96 well plates, selected, expanded and frozen in aliquots of about 500 to 1, 000. That was all done by our colleagues at Immerge Bio Therapeutics, Bob Hawley and Julia Greenstein. They were shipped to MU. We thawed them and used them for nuclear transfer. We did a total of 28 embryo transfers. I'm only going to list three here because the other 28, some of the pregnancies stopped half- way through. These three resulted in offspring. The top one, 212, was actually a mated surrogate. This animal was naturally mated, and then we did embryo transfer into her. Why would you want to do that? The pig requires a minimum of about four conceptuses to initiate and maintain a pregnancy, so we wanted to provide for the opportunity, in case there was really only one good nuclear transfer embryo in there, that it would have the opportunity to be carried to term. Seven animals were born. One of these was a nuclear transfer-derived piglet. It was a female, 0226. We transferred 92 embryos into her, and she had four nuclear transfer-derived piglets born in October. And number 230 had two nuclear transfer female piglets born October 15th.

This is the description of these piglets. Number 212-2 had an ocular defect with small ear flaps. It had a

decent size birth weight. Other than that, perfectly normal, healthy piglet. Number 230-1, it doesn't show up very well. There's supposed to be an H up there for healthy. She had a flexure deformity at birth and a little bit of right ventricular enlargement. Notice the smaller birth weight on this animal. Number 230-2, again a very small birth weight, the flexure deformity, died shortly following delivery of respiratory distress syndrome. Number 226-1 was normal, 600 grams, no significant findings. Number 226-2 had a flexure deformity again. It also had low velocity regurgitation at the center of the tricuspid valve. 226-3 died. Again a small birth weight. It seemed to be growing normally, but then during a routine blood draw at 17 days of age it died. It did have a dilated right ventricle with thickening of the heart wall. Number 226-4 had cleft palate, very small birth weight, 250 grams, died shortly following delivery of respiratory distress. There's three of the nuclear transfer piglets. For any of you that work with domestic pigs, these probably look ugly. These are the NIH miniature pigs. What's the behavior of our nuclear transfer piglets? Well, they interact normally with each other, they interact normally with caretakers, and they respond normally to stimuli.

The conclusions. Well, many different cell types can be used for nuclear transfer. The efficiencies are low. Part of the low efficiency might be a result of having these cells in culture for a long time and having them on the verge of senescence before we use them for nuclear transfer. And that was necessary to knock out the genes. Alternatively, or maybe in addition, these are miniature pig embryos that are transferred to domestic pig surrogates, and that may also cause some compromise in development. We're really not sure about that. We still have the large offspring syndrome, and I'll talk about that here in a minute. The evidence suggests that these aberrant phenotypes that are associated with large offspring syndrome are not passed on to the next generation. What's important to take home is that now we have the technology to make a specific genetic modification in the pig. Before we could add genes. Now we can take genes away. Now we can modify genes to get different proteins or different protein products.

What's large offspring syndrome? It was first characterized in cattle where they produced embryos in vitro. Simple oocyte maturation, fertilization, cultured to the blastocyst stage, embryo transfer, resulted in some calves that -- normal birth weights are 70, 80 pounds. Some of these calves had 200, 250 pound birth weights. So large offspring syndrome is characterized by species-specific phenotypes. In cattle it's large birth weight. We didn't see that in pigs. It's respiratory distress. In mice it's obesity, contracted tendons. I'll show an example of that in a minute. And again it was first described in animals produced by in vitro conditions. I want to cite a couple of interesting papers here. Conway, in '96, showed that the large birth weight in cattle was not inherited. They had two cloned bulls. One had a very large birth weight, one had normal birth weight, and they made it into a population of females. All the calves had normal birth weight. Just very recently Tamashiro published in Nature Medicine that obesity in these cloned mice is not inherited, is not passed on to the next generation. This is likely a result of methylation of the CPG islands along the DNA. I can go into some detail about that if some of the scientists have questions.

I kept alluding to this contracted tendon problem. Here we have number 230-1. At one day old you can see this front leg. It doesn't straighten out. By eight days of age you can see that this front left leg is now straight, and she's walking normally on it. Well, two of the ten domestic piglets and four of the seven NIH piglets exhibited this, and they did respond to physical therapy. Let me give you an example in pigs of the lack of heritability of this contracted flexor tendon problem. Earlier I described to you 4022. She was the transgenic pig that we cloned, and we made four clones of her. Well, she was actually the first pig, at least to my knowledge, that was actually an in vitro matured oocyte, fertilized in vitro, cultured to the blastocyst, and then transferred to a surrogate. She had the flexor tendon problem. She had contracted flexor tendons. In fact, she ended up getting an infection in her front forelegs, which was treated, and she's healed fine. But when we cloned her, only one of her four clones had this phenotype. In addition, she's now had 24 offspring, and none of them have had this phenotype. So it's something that is fixed in the next generation. The methylation pattern, for those of you that are familiar with DNA

methylation, the pattern gets erased during gametogenesis and reestablished, so you have the normal maternal or normal paternal imprint pattern. Large offspring syndrome is a problem for those of us in production agriculture if the cloned animal is our end point, but if we're interested in introducing a new genetic modification, the large offspring syndrome is a management concern, and it's a concern, but it's only in the first generation. I should also note at this time that there's a couple of groups that have reported cloned pigs that have not reported similar problems.

A large number of people collaborated to do this. I want to point out two important people, Julia Greenstein and Bob Hawley from Immerge Bio Therapeutics, who provided us with the genetically modified cells so that we could do nuclear transfer. And I also want to acknowledge Liangxue Lai and Kwang-Wook Park as the two people in my lab that did the nuclear transfers on a day-to-day basis. Funding for these projects has mainly come from NCRR, being an R01 to me, an R43 to Immerge, which is an SBIR. Funding also has come from a local program called Food for the 21st Century at the University of Missouri. I've also had funding from USDA-NRI.

This is a quote that Hans Spemann had back in 1927. He was describing embryonic induction, and I think it fits very well with where we are now. In fact, I've used it for a number of years. It works every year. What's been achieved is but the first step. We still stand in the presence of riddles, but not without the hope of solving them. And riddles with the hope of solution, what more can a scientist desire? Thank you.

DR. SALOMON: Thank you very much. That was really excellent. Again I think following the same pattern, if that's okay with everybody, if there are some specific questions that just speak to a clear message that has to be clarified right now, otherwise, what I'd like to do is do this whole sort of theme and then open it to discussion. Thank you.

DR. SWINDLE: I just have one question about the pigs that had congestive failure. Did they also have septal defects?

DR. PRATHER: I don't remember seeing it. Mostly it was just enlargement of the ventricle. In fact, in one of these animals the ventricles got so large that it pushed the lungs out of the way, and it would just start panting, and it actually distended the ribs a little bit.

DR. SWINDLE: But you were diagnosing by echocardiography?

DR. PRATHER: Yes.

DR. SWINDLE: And you were seeing right heart failure and coronary hypertension and stuff?

DR. PRATHER: Correct.

DR. SWINDLE: All right. Well, I'll talk to you later about it.

DR. PRATHER: I should also point out that I'm the reproductive physiologist. I had another guy that was responsible for that, so I don't know if I can technically answer.

DR. SALOMON: Always fix blame and take credit.

DR. PRATHER: Well, I tried to spread the credit around a little bit.

DR. SALOMON: Louisa?

DR. CHAPMAN: I have a question. You mentioned several piglets had died within about a month of birth of congestive heart failure. Is it appropriate to assume that that is something unique to this cloning or is that something you see in pigs?

DR. PRATHER: No. It's something that's a result of the cloning procedure. In the calves they've seen the same thing. Many of these animals get respiratory distress, they start panting, and then they'll succumb.

DR. CHAPMAN: How often do you see something like that in just normal run of the mill regular pigs?

DR. PRATHER: Very seldom. This is a result of the cloning procedure, but if you can get these animals to adulthood and they can reproduce, they should not pass that on to the next generation. One of the concerns that the public has asked me about is you want to use these for hearts. The hearts aren't any good. Well, if you can get them to puberty, these animals aren't going to be used for anything other than mating purposes anyway. They only have one copy of the gene knocked out. You're going to have to get them both knocked out to have the absence of that cell surface molecule. So if you can get them to where they reproduce, their offspring are going to be normal. At least all indications are that they will be.

DR. SWINDLE: I can answer your question. In all probability they do have either patent foramen ovale or high membranous septal defects. We have seen it in the inbred lines of pigs, and it's shown up in necropsy studies. If you take a look at the Yucatan, they had, when they started off, flexure tendons when they started to line breed the Yucatan miniature pigs. They also had ventricular septal defect and patent foramen ovale. It's not uncommon. The symptoms are exactly as you described, early onset of respiratory distress, right heart hypertension. Some of them can be very tiny sorts of things. It's polygeneic as far as we know, but I suspect that that's what they encountered because it's been encountered in other lines that have nothing to do with cloning or transgenic production, just line breeding.

DR. PRATHER: They see it most in the nuclear transfer cloned offspring. These animals did not have a patent foramen ovale.

DR. SWINDLE: They did open the hearts and look for them, the one-millimeter ones? Because it can show up in about a third of the population of some line bred pigs.

DR. CHAPMAN: So am I understanding you correctly that you're saying this is the equivalent of the kinds of congenital problems we see in inbred human populations?

DR. SALOMON: I don't think that's what they're saying because the inbred is forever after that, so it's not that.

DR. SWINDLE: Yeah. What I'm saying is exactly what he's seeing shows up in nontransgenic line breeding animals.

DR. PRATHER: Yes. Some of this does show up in normal animals, but the percentage that you see it is much lower.

DR. SYKES: Also this certainly isn't obligatory in inbred pigs. Our MGH miniature swine have been inbred to a coefficient of homozygocity of 94 percent to the point that they are immunologically unresponsive to one another, and these animals, as far as I know, don't have any problems like that.

DR. SALOMON: Dr. Wright?

DR. WRIGHT: The size variation that you pointed out is really quite striking. I was wondering if anybody's looked at placental pathology as maybe an explanation. I mean you follow these implantations going on and some from two different sources. So were the placentas examined?

DR. PRATHER: Yes, the placentas were examined, and other than them being small, there wasn't anything grossly wrong with them. We did histology on them, and they looked normal, unlike what you see in cattle, for example, where you have the -- it's a different placentation system. It's more of a diffuse general. It covers the whole surface of the placenta, whereas, for example, in cattle and sheep you have a specific area of implantation or attachment. And at least in cattle the implantation sites get very large, and there's very few of them. This is different than that.

DR. WRIGHT: And the flexor tendon problems, that's not anything like club feet in people? I mean that's something you get with oligohydramnios just if there isn't enough amniotic fluid.

DR. PRATHER: You hear the example that these animals, at least in cattle, the original thought was that the birth weights were so big they were just crowded in there, and that's what was causing it. These pigs are little. There's not any crowding problem in there.

DR. SALOMON: Okay. I think that it would be great to go on to the next talk, which is Genetic Modification of Gal Transferase in Miniature Swine. This talk will be given by Dr. Julia Greenstein from Immerge Bio Therapeutics, Inc.

Agenda Item: Genetic Modification of Gal Transferase in Miniature Swine

DR. GREENSTEIN: I'd like to thank you for the opportunity to present the remainder of our program. Randy gave you a wonderful introduction and sort of finished to where we got to. I'd like to tell you first about why we chose to target this gene loci and then how we generated the cell lines that got those pigs you've already seen. Immerge Bio Therapeutics is a joint venture between Bio Transplant and Norvartis, and our goal is to generate pigs and a treatment paradigm much based on the work that Megan Sykes so eloquently described today, to use those two things together to allow the use of porcine cells, tissues and organs to treat serious human diseases. You've heard a lot also this morning about the various steps in the immune response to pig in both nonhuman primates and in humans that define the problems that we are attempting to address. They're conveniently divided into three areas of hyperacute rejection, delayed vascular rejection and cell-mediated rejection. It's really the first two that drove us to the need to try to knock out alpha-Gal transferase locus.

Hyperacute rejection, as you know, is a result of discordant xenotransplantation, pig to nonhuman primate, done across a species barrier that's defined by the presence of this natural antibody. In the pig-to-primate combination the vast majority of that antibody is specific for a terminal galactose alpha 1,3 galactose linkage on both glycoproteins and glycolipids of probably every cell in the pig. Hyperacute rejection was attempted to be alleviated in a number of ways once we understood the specificity of those xeno-reactive antibodies binding to the endothelial cells of the graft. Antibody binding mediates complement lysis, and you've heard a lot this morning about how we've tried to basically take these effective functions and inhibit them and ignore the presence of the antibody binding to the epitope. That's met with some level of success in what we might conclude as eliminating hyperacute rejection, but the basic phenomenon we have not eliminated is the ability of the antibody to bind to the antigen. As we accumulate more antibody binding activity, we believe that we see loss of these organs by delayed vascular rejection, so although the mechanism of rejection is distinct, the mediator of that rejection still continues to be the alpha-Gal antibody binding to the Gal epitope on the endothelium. And you heard

today about how that endothelium is activated and how downstream it can cause a series of very detrimental events to the transplant and result in early loss of that transplant. Subsequent to that our hypothesis is if we can get rid of the antibody-mediated events, the cell-mediated immune response will get us as a third prong to the immune system protection of the individual. It's mediated by T lymphocytes. Megan went over these facts in great detail this morning, so I'll skip through it very quickly, but just to remind you that T cell cell-mediated rejection is the primary rejection pathway today in human-to-human transplantation. And again as Dr. Sykes alluded to, the T cell reactivity in man to pig cells is much greater than that to allo reaction, and we know that we don't want to use too much chronic immunosuppressive drug therapy because of the side effects of those approaches, so we look forward to putting together the genetically modified pig with a tolerance induction program.

The molecular advent of transgenesis in the pig, as you've already heard about today, allowed us to in some ways declare that hyperacute rejection was bypassed. That work was done by a number of groups, and you've seen today many studies using hDAF and even complement regulatory gene transgenic pigs, which showed that we could sustain the organ through what would have been hyperacute rejection and to start to see two weeks to one-month survival of various organs. That approach was also further modulated by trying to remodel the carbohydrate structures. So could we add other sugars to basically block that terminal alpha-Gal transferase activity in the pig? And again it looked like there wasn't enough complete blocking of the alpha-Gal epitopes, so you still see antibody-mediated rejection events in what are called carbohydrate remodeled cells. So we get left with all the rejection pathways really leading to the antibody specific for Gal alpha 1, 3 Gal. We worked along a path under the assumption that we really had to be able to remove the alpha-Gal transferase completely from the genome of the pig in order to control early graft loss using these cells or tissues as transplant modalities. This cartoon just shows you the carbohydrate structure of alpha-Gal 1, 3 sugars. You can see that it's very similar to blood group A and B, which is shown next to it. We've tried transgenic approaches, as we've already discussed. Many groups have tried specific immunoabsorbent columns, columns that took out all the antibody from the recipient, soluble forms of the sugar, all bypassing hyperacute rejection, but not giving us enough control over the antibody response. So we wanted to knock out the gene. We wanted to have a permanent solution to the problem of Gal-specific natural antibody. We also wanted to set up a program of modifying the pig so that we could avoid any unnecessary recipient treatment possible. This therefore would leave the recipient natural antibody intact, and we wouldn't have to worry about it in terms of the transplant. We knew at least the biological feasibility of being a Gal knockout was already demonstrated, first by all the people in this room, all world monkeys and man, and maybe more importantly, in terms of making the knockout in the Gal knockout mice that were described by John Lowe. Then you get to the problems of the pig.

So probably almost ten years ago we started having these discussions, way before anybody would have even believed that one could take nuclear transfer from either an adult or fetal cell, genetically modify that, and end up with an animal like Polly or Dolly. So our first naive approach was well, maybe there's a pig out there that has low expression of Gal transferase and therefore low epitope. Well, we went through the entire MGH inbred herd. We looked at other pigs. I don't think anybody ever convinced themselves there was a low expressor line. We thought about homologous targeting by direct microinjection and transgenesis, which has been shown to be possible in the mouse. The frequency of the event is very low. We actually attempted a study of trying to take alpha-Gal transferase out of the mouse using direct microinjection and transgenesis, and we were unsuccessful, so we convinced ourselves before we set out on that folly that the frequency of the event was unlikely to be able to succeed in large animals. We then had an extensive program, both in collaborator labs and in our own labs, to try to make a pig embryonic stem cell. Again as Randy already pointed out to you, most people who are familiar with genetic modification in the mouse say, oh, it's easy when you have embryonic stem cell lines. What's your problem? Well, we and many people have walked down the field of trying to get a porcine embryonic stem cell. To date there's been no convincing animal embryonic stem cell defined and used to generate an

animal from that cell line.

Seven years ago, in 1995, the first production of sheep from nuclear transfer cloning convinced us and many other people that we now had a methodology in hand that perhaps would allow us to make the alpha-Gal knockout in the pig. We found ourselves spending a lot of time with pig embryologists, and our collaboration with Randy Prather has really been very rewarding for both groups. Pig reproductive biology is distinct from the species where nuclear transfer was first successful, so we had to learn a lot about pig reproductive biology to get even the simple unmodified pig cloning program to work. Once that happened, we then had to be able to target this gene loci, so we wanted to target the alpha-Gal transferase, and we knew that it required homologous recombination approaches. The first thing to do was actually to first clone the pig alpha-Gal transferase that was done with the collaboration of Kent Gustavsen at the University of London. Based on that genomic clone, we first studied the inbred miniature swine to make sure that they were not polymorphic at that locus, to make sure that we could really design a homologous recombination vector that would work in this background. We convinced ourselves that the DD herd that we were most interested in did not have any genetic polymorphism at the alpha-Gal loci, so we set out to design a vector containing a very long homologous stretch and putting in stop codons, so putting in new nucleic acids into that vector that would cause, if it got put in the right area of the enzyme, cause that enzyme to basically stop in the middle of the catalytic domain and would not allow a full length RNA to be produced from this DNA so that therefore you wouldn't have any active enzyme when you translated it into protein. We also had to define conditions under which we could culture fetal fibroblasts from these pigs, transduce those fetal fibroblasts, select them and analyze them as quickly as possible because these cells will senesce. If the cells senesce in culture, you will not be able to make a pig from that cell line. So everything had to be done quickly and efficiently.

We started out with our desired strain, and we made fetal fibroblasts. The targeting vector was then added to those fetal fibroblasts. We had to have a way to select those cells, so we put a drug selectable marker into that vector as well. The cells were transfected and cloned and selected. This is a cartoon of the vector. It has 23 KB homologous to the alpha-Gal transferase gene in the pig. That's a very big targeting vector. You can see that in the center there's stop codons put in and also a neomycin gene. That's something to think about as we talk about regulatory issues with animals derived in this way, and I'll highlight it later, but suffice it to say you should remember that there is now a drug selectable marker in this cell line which will become the pigs that you've already seen pictures of, even if they are funny looking.

Okay. Now for the hard work. We knew that the homologous targeting event was a very low frequency event, so the clones that resulted in the pigs you've already seen started with ten to the eighth fibroblasts in 10, 000 separate wells. Those fibroblasts were transfected, and they were selected for approximately two weeks under the selectable marker, which is an antibiotic. From those 10,000 wells, 200 stable clones, so 200 of the 10, 000 wells had cells that were growing in them. And we had to figure out which of those 200 stable clones had the appropriate genetic modification, so we first did some PCR analysis of each of the 200 stable clones. We got ten candidate clones that looked like they had been targeted for alpha-Gal. We confirmed the targeting event in those ten candidate clones by genomic PCR, and the result from the initial ten to the eighth fibroblasts that we started with was five potential knockout donor cell lines. When you put that kind of analysis together, it's just good to realize, when we start talking about multiple targeting events or multiple genetic modifications that were suggested over the course of just this morning's discussion, one has to remember how difficult it is to make the right cells to get this whole process started. So this is a summary of four separate fetal cell lines that were transduced and selected, and you can see at the bottom the average of the four experiments that were done, and you can see on the order of one in ten to the seventh cells ends up having the appropriate targeting event and can then be tested for the ability of that cell line to be able to be used as a nuclear donor and do nuclear transfer with it. Randy already showed you this table. You can see that in this case, excluding the mated

surrogate which Randy took you through, in 20 transfers to recipients over 2, 000 embryos were transferred to those 20 recipients. We had six pregnancies, two which went to term, and we had four viable piglets from that process. This is the PCR analysis of the piglets that resulted from those pregnancies. We've tested all seven of the piglets that Randy talked about already, and the PCR analysis basically shows you, in the middle panel, the probe is designed so it picks up both the wild type and the targeted allele. You can see that each of the knocked-out animals has both the presence of still a wild type allele, but also has the targeted allele as well. And then in the bottom panel this is a blot which will only pick up the targeted event, and so you can see that each of the targeted piglets that were born had only in that case the presence of the targeted allele because the wild type was not probed for there.

There's been much discussion about whether we did or didn't have a southern analysis in that wonderful journal, The London Times, over the controversy between what happened between our publication and the group in Scotland, PPL. This is the southern analysis of the six animals, and you can see again by southern analysis that we have knocked out the gene. It picks up both again the wild type and the knocked out gene. That's a picture. We don't really think they're ugly. We think they're kind of cute. This animal has one allele of the Gal transferase removed from its genome and will serve as the core founders of generating the double knockout pig. So we have at this point four healthy females. They will reach sexual maturity at between six to eight months. They have one allele missing. There is no expectation, based on what we have done in our cell line analysis, that there would be any change in the alpha-Gal transferase expression in these pigs, so we haven't actually even looked. The problem is, now that we have four females, there are a variety of approaches to homozygous animals which have a combination of either having to wait a long time or having to solve some other technical problems in the approach to making a homozygous animal. There is of course, as already mentioned, some risk that these animals, when they are homozygous as the knockouts for the alpha-Gal transferase gene could have some aberrant effect and could even be lethal. Again we go back to the mouse and man to feel comfortable that we will be able to get the homozygous knockout animals born and healthy.

This is just to take you through the three approaches that we're actually currently undergoing to make the double knockout pig which will be the donor of some interesting organs to test in the primate models. First, if you only have a female founder which is Gal T negative Gal T plus, one allele knocked out, you can breed her to a normal male who has two copies of the Gal transferase gene, and they're both normal. In that case 50 percent of their first generation offspring will have one allele knocked out, again not interesting biologically, but important in the process to get to the double knockout. You then from that F1 generation have to now wait for that series of animals to reach sexual maturity, and you brother- sister mate the two animals in this picture that have the one gene knocked out. Of course, you have to hope that you have a boy and a girl in that first litter. So those animals which have one allele knocked out, both male and female, you breed those, and again only one quarter of those animals will be double knockouts for the Gal transferase gene. So you have to spend a lot of time waiting for the two generations of pigs, and you also only get 25 percent of your animals that have the phenotype that you want to use for your experiments.

The other more straightforward approach is to produce a female and a male founder, and we're currently trying to do that. In that case you'd save yourself one generation, and you get still only 25 percent of the offspring as the double knockout, but at least you get it one year earlier than the slide before. The fastest but most technologically risky approach is to try to re-knock out that remaining allele and re-nuclear transfer clone the animals. We can take founder animals and actually take fetuses rather than those four animals that we're taking very good care of while they reach sexual maturity. We could take a pregnancy that had nuclear transfer fetuses in it where we had targeted one allele and make another cell line from those one allele targeted fetuses. You can then isolate those cells, and now you have to make another vector. You can't use that vector we spent so long optimizing because the cells are already resistant to that antibiotic allele. You have to design another allele and select those cells under different conditions,

but it is theoretically possible to make that cell line, which now has no functional copy of the Gal transferase, and then send it back to Missouri and re-nuclear transfer from that double knockout clone, in which case now every animal that is born from those cells should lack the ability to make Gal transferase completely. Those are the three ways, and we're trying all three.

So in summary, nuclear transfer cloning allows you to introduce complex genetic alterations, either gene knockout or gene addition, without having embryonic stem cells. It doesn't require a large herd of embryo donor animals. As Randy alluded to, the recipients for these animals are outbred pigs. The oocytes that we use for nuclear transfer cloning of these animals are also from outbred pigs. It allows shortened time frames for animal production, and relevant perhaps to the regulatory discussion, there will be, at least by these methodologies, antibiotic resistance genes carried in the pigs and in the organs from these pigs. Just to give you a little bit more of a picture of the swine that we've chosen, the MHC inbred miniature swine were first selected to be able to do organ transplant experiments between known histocompatibility-type differences in the swine. They are also, because of their size, better matched for donor size for human transplantation than domestic pigs. At full adult weight they weigh between 200 and 350 pounds compared to 1, 000 pounds of a domestic pig, and it's very important to match organ size in terms of choosing organs for transplantation. They are fully inbred at the major histocompatibility complex and largely inbred, as Dr. Sykes already explained, in the rest of the genome. This obviously gives us some quality advantages in terms of preparing animals for clinical use. They've also been maintained over the past 30 years in a research environment and have been fed a vegetable- based diet, which again has both infectious disease advantages and quality advantages in terms of the source animals that would be used for transplantation. It also turns out that at least some of them have an advantage in terms of porcine endogenous retroviruses. We went through our inbred lines and tested them for both pig and human tropic porcine endogenous viruses. I want to be clear about this. All of the lines produced pig tropic PERV. A lot of times when we talk publicly, people make the assumption that some of these pigs are PERV free. To date none of these pigs are PERV free, but we have identified lines within this herd that have a low frequency of human tropic PERV, and within those low- producer families we have identified animals that lack the ability to produce human tropic PERV. That was recently published in Journal of Virology. So it is those animals that lack the ability to produce human tropic PERV in culture that we will derive our donor animals from. In terms of the remainder of the herd it's interesting to note that the human tropic PERV, when we started characterizing it, was the only recombinant virus that was found. Either PERV A and PERV B are the two subtypes of PERV that can infect human cells. We have not seen evidence of a full- length competent virus, either PERV A or PERV B, from these miniature swine, but we have detected recombinations between PERV A and PERV C, which can form viruses that can infect human cells in culture. So it's possible that these miniature swine, which originally were derived from two animals, lack replication competent loci, and therefore, that recombination is a prerequisite for replication in the viruses in these herds.

So what I've told you about today is a big collaborative effort to produce founders to generate miniature swine incapable of expressing Gal alpha 1, 3 Gal epitopes. We also succeeded in the first demonstration of a nuclear transfer gene knockout pig and the first demonstration simultaneously of nuclear transfer cloned miniature swine, which was important to our goals. This work has been an active collaboration between a number of groups. Randy Prather showed you the list of people that have been involved in this program. The excellent group that we've been able to collaborate with in Randy's lab has really led us to this success. We have been studying these pigs and tolerance induction programs with the Transplantation Biology Research Center at Harvard Medical School, and it is really the animal genetic engineering group led by Robert Hawley that produced the cells for this program, and the PERV work that I alluded to is out of our safety group run by Clive Patience.

Just to finish up, it was particular fun to be the target of some comics in The Boston Globe when we published our paper in Science. See, it's not really an ugly pig. It's kind of cute. Thank you.

DR. SALOMON: Thank you, Julia. Are there some specific questions of clarification or should we wait for discussion? Alan?

MR. BERGER: I was just curious. There was a comment made this morning about a cause and effect. When you do a Gal knockout pig, are there any effects to the pig that change the physiology in any way or anything that you're testing or looking down the road that might actually cause another problem?

DR. GREENSTEIN: We don't really know what to expect until we have the double knockout. We can go to the mouse and to primates and say that it's at least a gene that's not required for any essential process in either of those species. You can really argue it either way. The pig expresses more Gal epitope than the mouse, so we've had discussions with people who predict that there will be a problem in knocking out the second copy of the gene, but there's really no way to predict it until you try.

DR. SALOMON: Okay. I'd like to introduce the third and ultimate speaker of this particular theme, which is Guerard Byrne from Nextran, who will talk about the Development and Characterization of Transgenic Pigs for Xenotransplantation, showing us a little bit of their strategy for doing something similar.

Agenda Item: Development and Characterization of Transgenic Pigs for Xenotransplantation

DR. BYRNE: Dr. Salomon, thank you. Thank you to the SACX committee for the opportunity to speak here today. We've just heard two very interesting talks about the newest technology for making transgenic pigs. I'd like to take you a step back from that scientific forefront and provide you with a review of the state of the art for pigs made by pronuclear microinjection. This is the topic that the committee asked us to speak on. Since the technique of microinjection has been around for over 20 years now and has essentially remained unchanged throughout that time, I'm not going to review in detail how that's done. Instead I'm going to focus on some of the lesser known problems that arise in the development and breeding of transgenic animals and call your attention to techniques that we've found useful for characterizing animals prior to preclinical use. In the latter part of the talk I hope to give you a flavor for the impact that these transgenic animals have had on xenotransplantation.

So to set a historical perspective, let me just remind you that the very first transgenic pig that I'm aware of was produced by Hammer and colleagues and published in Nature in 1985. This was an animal expressing a metallothionine growth hormone regulated gene, and it paved the way for the development of a large number of transgenic animals to date, animals expressing a variety of growth regulatory gene products, expressing genes with potential pharmaceutical application to be secreted in the milk, expressing genes to enhance disease resistance, and expressing animals that we've had experience in, animals producing human hemoglobin as a source of hemoglobin for cell-free blood substitutes, and of course animals expressing complement regulatory gene products and galactosyltransferase, which you heard of today, for xenotransplantation. Nextran in its original format was a biotech company called DNX, and we published our first transgenic pig in 1980. Since that time we have produced over approximately 200 transgenic founder pigs, the majority of those targeted to complement regulatory protein expression or Galactosyltransferase expression.

I can almost skip over this slide. We've had such a nice introduction of things. Microinjection is a relatively mature technology. Approximately one to ten percent of the animals that are born retain the DNA that's been microinjected, and this number can vary quite wildly depending upon the type of DNA you're injecting. This technique can only add genes, and that is an important limitation. The genes that are added integrate at random sites in the genome and typically go in as multicopy concatamers. I'll have a lot more to say about this issue later in the talk. Microinjection into the pronucleus cannot engineer gene

knockouts. In comparison, the nuclear transfer techniques that we've just discussed are an emergent technology with a relatively low efficiency, and in this case efficiency is not the number of animals born that have the genetic modification, since essentially all of them are clones and all retain the modification that were designed, but the efficiency is based on the frequency with which a reconstituted embryo can actually survive to term and produce a live offspring. Like microinjection, the tissue culture techniques that are used prior to nuclear transfer can introduce genes randomly, as in the case of microinjection, or using selection schemes that derive rare homologous recombination events, they can select for genes that occur as single copies at targeted sites, and accessibility, which has endowed it with the ability to make knockout mutations.

When you start to make transgenic animals using pronuclear injection, the first thing you start with are the constructs. I've illustrated here the design of three broad classes of constructs, and I've done so because each of these types of constructs have given rise to transgenic animals which over the years have made important impacts on xenotransplantation. The simplest type of construct is what I'll call a cDNA construct, and it places the coding capacity of a gene between 5 prime regulatory and 3 prime splice polyadenylation signals. These genes are typically quite small, 2 to 5 KB, easily integrated into the genome, but their small size and their limited amount of information often limits the level of expression and the authenticity of their expression pattern. So you can make a lot of animals with this DNA, but many of them are not very useful. A more sophisticated design is the development of a mini gene. This mixes the combination of genomic DNA encoding 5 prime regulatory sequences and intron sequences with cDNA sequences. If these genes are well designed, they can be very useful in providing highly authentic patterns of transgene expression. And finally, for those of us who don't know anything about the gene we want to inject, we use large genomic fragments under the assumption that these large pieces of DNA will contain all of the 5 prime and 3 prime regulatory elements to provide good gene expression. And indeed although these genes tend to be quite large and can be difficult to introduce intact into a pig, when they are introduced that way, they provide very good levels of gene expression.

So the process of microinjection begins with a fertilized pig egg in which a glass needle is inserted into the pronucleus, and DNA is released into the pronucleus. A series of those eggs are transferred into foster parents, and then in about four months litters are born if you're lucky, and one to ten percent of those offspring will retain the DNA. As you heard Dr. Prather indicate earlier, there's a certain level of chimerism inherent in this generation, and that's frequently seen. Subsequent to that it takes approximately a year for that animal to mature and breed up another generation of G1 animals, and it's really these animals which are the first animals that you can use to address whether the modification you've made is useful in terms of gene expression and also whether it is useful in terms of xenotransplantation. So this whole process takes about a year and a half from designing the gene to actually getting an answer in terms of transplant survival.

Research in xenotransplantation has been dominated by three lines of transgenic pigs that I've illustrated here. And I don't mean to leave anybody out, but for the most part the vast majority of pig-to-primate transplants have been done using either a human MCP genomic construct, which we developed at Nextran and was published by Lisa Diamond, or using the hDAF transgenic animals which contain a mini gene for DAF, and that's been described by Cozzi and colleagues and was developed by Imutran. In addition to these two constructs, there is a class 1 regulated H2-DAF beta actin CD59, a cDNA-containing transgenic pig which has been described and used in some pig-to-primate transplants, but more importantly, has been used in ex vivo liver perfusions, and that work was previously alluded to and published by Marlin Levy at Baylor Institute.

Clearly I can't go through all of these animals and give you a detailed blow by blow of everything involved in them. Instead what I'd like to do is concentrate on some of the problems we've identified over the years at Nextran in the pig lines that we've used. Most of these problems center around the

concatameric integration site and the stability of that integration site, so I'll spend some time on that, and then secondarily, I'll spend some time looking at assays to look at the stability of gene expression, not only in the first generation, but in subsequent generations. All of this is of course designed to ensure the reliability of a transgenic organ for clinical use.

When DNA is introduced into a pronucleus and subsequently integrated, it generally integrates as head to tail concatamers which I've illustrated here. These concatamers may express the gene either in each individual concatamer giving rise to RNA which contributes to gene expression, or alternatively, one or a few of those concatamers may be responsible for all the gene expression that you see. This would be an interesting academic question and is still an open question as far as I know except for the fact that on occasion transgenes will integrate in such a way that there are head to head and tail to tail junctions. This type of structure is not infrequent and can lead to instabilities, one of which I illustrate here. So it's possible for this copy of the transgene to fold up, anneal to another copy upstream of it, and this structure then might resolve by deleting the intervening loop and reducing what was a transgene integration site with six copies to three copies. This may or may not affect the overall expression detected in the animal line. An example of this phenomena which we identified quite some time ago is illustrated here for an animal that was designed to express human hemoglobin using a mini gene type of construct. So it has a regulatory sequence here hooked up to a human alpha globin gene and then following, a human beta globin gene.

The original founder animal here and in the middle of this southern blot analysis showed a restriction pattern composed of four bands of 13, 5, 4 and 3.2 KB. His offspring also showed that pattern, but on occasion showed a pattern which was missing the 4 KB band. In this system the expression of this transgene is analyzed by looking at blood samples and using isoelectric focusing to resolve alpha beta heterodimers. On the bottom of this slide you can see in human blood there is just a human alpha human beta dimer. In the founder pig that human alpha human beta dimer is present, it's well resolved from the pig alpha pig beta dimer, and in this system a heterodimer consisting of human alpha and pig beta is also made. What you can see is that across the litter of animals those animals which inherited the original restriction pattern here also show human alpha globin expression, but those animals which now have lost the 4 KB band show no expression of human hemoglobin at all. This is an example of where the instability of the locus can lead to loss of transgenic expression.

We've also seen this type of phenomena in a complement regulatory system, and here we've used a different type of gel analysis to directly examine the integrity of the integration site. This is an electrophoresis system called a CHEF gel system, which is capable of resolving very large pieces of DNA. For this analysis what we did was cut the DNA prior to electrophoresis with an enzyme that did not cut within the concatamer to resolve the entire integration site now as a single 80 kilodalton band. In most offspring in this slide that was the band that was inherited. In some offspring -- and I've grouped a whole set of them here -- that 80 kilodalton band shows a deletion, reducing the size down to approximately 50 KB in this worst case. Unlike the previous example, however, these deletions did not seem to affect transgene expression, as illustrated here looking at a fax analysis of peripheral blood lymphocytes from intact animals stained for CD55 and peripheral blood lymphocytes of animals containing the deletion stained for CD55, and essentially they have virtually identical expression patterns. So the concatameric integration site can lead to instability in the founder, and when it is passed on, if it's stable, it's passed on and maintained in that format. If it's unstable, those animals which inherit a rearranged version, they then stably transmit that rearrangement for generations down the line.

The other issue that is important in characterizing these animals is to look at expression patterns. Let me illustrate that for you examining a line of animals that we've used a lot in transplantation. This is a human CD46 or MCP expressing line. This line contains a large genomic fragment of approximately 60 KB that contains all of the introns and exons of MCP. We selected this type of gene because expression of

complement regulatory genes, as was pointed out earlier, is not an insignificant amount of expression. These genes are expressed at high levels in a wide variety of cells, essentially all cells exposed to serum. So in order to make a complement regulatory organ that will affect hyperacute rejection, it's important to get a high level of gene expression. That's illustrated here in this northern blot which compares human expression of MCP in various tissues; heart, kidney, liver, lung and spleen, to MCP expression in this transgenic organ, showing you very high levels of gene expression. Additionally this gene expression shows an authentic pattern of expression, as I illustrate here in this liver biopsy stained for CD46 expression in a transgenic pig and CD46 expression in a human. And again you see that most of this expression is concentrated around the vasculature.

So the question we wanted to ask is given the founder animal and having the F1 generation breed, how stable is that gene expression pattern within this horizontal area? So between litter mates how consistent is gene expression? Then given that we're going to go to another generation, how consistent is the vertical stability of gene expression, that is, the stability of expression from generation one to generation two and so forth?

Now, because of the size of this transgene and because it is there in multiple copies, it's difficult to get a direct read on the stability of the system. What we've done instead is to take probes from the immediate ends of the microinjected DNA and do southern blot analysis of tail DNA to generate a fingerprint for each of these probes. The reasoning here is that if there's a rearrangement within the locus, these end pieces should be involved, and if rearrangement occurs, one of these bands should disappear. What we see instead is that throughout the G1 and comparing G1 animals to G2 animals, both the 5 prime and 3 prime probes show a very consistent restriction pattern. So at this level of analysis there's no evidence of any gene rearrangements going on, although obviously that has to be supported by a continued analysis of this line. We also examined the RNA expression of MCP in G1 offspring and in G1 and G2 offspring in the heart, kidney and spleen, and see again a very consistent pattern of gene expression in this line.

So to summarize, producing transgenic animals by pronuclear microinjection is an inefficient process. Over the years Nextran has produced approximately 140 xeno-related animals. Of these, only 10 or 12 lines proved useful. Now, the efficiency of this process has to be defined. That is for us a line was useful only if it began to pass a bar of performance. If we already had a line that had a higher level of gene expression than the new one that came along, the new one wasn't really interesting to us. I'm not trying to suggest that only 10 or 12 lines expressed. What I'm trying to say is that as you go through this type of experiment, the level of performance that you want, the bar that you set for each transgenic line, gets progressively higher. Under favorable conditions it can take from six months to a year in order to identify whether a transgenic animal is useful or not. If that founder animal has some of the problems that I illustrated for you earlier, then that problem can add many more months, even years, to that analysis.

We have spent a considerable amount of time injecting very large genomic constructs, and although there are publications in the literature on transgenic mice of animals containing 200 and 300 and higher kilobases of integrated DNA, at least in our hands we find that it's very difficult to integrate intact a piece of DNA that's greater than 100 KB. Some of these issues can be addressed using the new technologies of transfection and nuclear transfer. Part of the instability inherent in pronuclear microinjection can of course be directly addressed using nuclear transfer because you can select for homologous integration at defined sites, so now you have single-copy integrations, and you have integrations in loci which are unlikely to rearrange. So that's an important characteristic, but I would point out that the selectable marker required to select these rare events would then have to be removed, and it's not clear in these primary fibroblasts if that marker can be removed efficiently or not. Also nuclear transfer and the transfection technique and tissue culture are easily more adaptable to large pieces of DNA, so integrating larger genomic fragments or even fragments from yeast artificial chromosomes should be more doable in this type of system.

So what impact have these animals had on xenotransplantation? First I would say that the complement regulatory transgenic animals were never designed to be a panacea for all of our problems in xenotransplantation even though I think secretly we all hoped that they would be. These animals were designed specifically to address issues of hyperacute rejection, and as such I think they've performed very well. Experimentally they've been a great aid to us in doing pig-to-primate transplants, especially when you consider the alternative, the alternative being a systemic inhibition of complement using a variety of methods, cobra venom factor or soluble CR1. Technically that's easy to do except that it creates an animal in which you have a high level of immunosuppression, a lack of complement, and invariably that aggravates what is already a difficult situation of controlling problems with infection and drug toxicity.

Up until a few years ago I would say that what appeared to limit the success of xenotransplantation, and of course what the complement regulatory pigs were never designed to address, was the inability to control and induce anti-Gal immune response. An example of that response is illustrated in this slide. I took this figure from the publication by Marlin Levy on two patients that had received ex vivo liver perfusions. What happened to these patients, this patient received an ex vivo perfusion for approximately ten hours and then was bridged to an orthotopic liver transplant. So here is an example of a patient who's clinically immunosuppressed, and after a very short exposure to pig tissue, shows a dramatic increase in anti-Gal IgG and IgM in circulation, and that increase was persistent for essentially a month and a half or so. It's this induced antibody which is thought to underlie many of the problems associated with acute vascular rejection.

So how do we get around this? You just heard about the development of the Gal transferase knockout pig. Certainly one way of getting around this is to reduce the antigenicity and to produce an ideal pig, an ideal pig consisting of a Gal transferase homozygous knockout and some human complement regulatory protein. We have also heard this afternoon suggestions of integrating additional capabilities into what would be called an ideal pig, capabilities including coagulation regulation, abilities to inhibit endothelial cell activation or apoptosis, the incorporation of cytokines or human cytokine receptors, or the secretion of T cell costimulation blocking agents. I would just point out that this ideal pig would take a very, very long time to produce given the types of problems that I've illustrated to you before. As evidence of that, here I've shown you just a simple breeding scheme of what it would take to incorporate complement regulatory function into a Gal homozygous knockout. This scheme is actually quite ambitious in the sense that it's already assuming that we have both a male and a female heterozygous pig for the Gal transferase knockout, and I don't think that situation currently exists. But if we had such animals, we could breed them to make a homozygous animal, breed that animal with the complement regulatory animal with the transgene to get a heterozygote and transgenic CRP animal, and then backcross that to a homozygous animal to finally get the Gal transferase knockout CRP. This entire process with just using a simple breeding scheme takes over two years. So to integrate one gene into this type of background takes two years. To integrate additional genes, let alone to develop those transgenics, would take a very long time. As Dr. Greenstein showed, this process could be significantly shortened using nuclear transfer techniques, but there are a lot of scientific uncertainties about that process.

An alternative way to control the Gal immune response was initially discussed by Dr. Zhong earlier this morning, and that is the use of these alpha-Gal polymers to block anti-Gal antibody in vivo and to block the induction of anti-Gal antibody. Nextran has produced one of these polymers which we call 1285, and this is just an example illustrating the effect of those polymers. In a baboon which is undergoing a normal transplant, the anti-Gal antibody for IgM and IgG shows a very strong induction when the organ is rejected and removed, suggesting that there is a very intense immune response in the presence of that organ, which leads to this increase in anti-Gal antibody in circulation. So for IgM it's a two and a half-fold increase. For IgG it's a greater than 50-fold increase. In the presence of these alpha-Gal polymers, however, when the organ is rejected at this point on day 20 and then the polymer is no longer

given, the level of anti-Gal IgM and the level of anti-Gal IgG does not rise to the original baseline levels. Under these conditions we can dramatically limit the impact of anti-Gal antibodies, and that has led to the observation that organ survival is being prolonged.

I've illustrated for you some results that we've recently had using human MCP in cardiac xenografts where organ survival has now moved out to 47, 53 and 109 days. Under normal conditions the average heart might have lasted say 25 or 35 days, so for the first time we're pushing out beyond that one-month window into the two and three-month window, and I think this is important progress. At the recent xenotransplantation meeting Manes, et al. reported very similar results using the hDAF transgenics, and in this case I would hasten to point out that none of these animals actually rejected their grafts, so at 60 days they had no rejection whatsoever, but the animal succumbs to infection and postoperative complications.

So to conclude then, the efficiency of xenotransplantation initially appears to be limited by an induced anti-Gal response that overwhelms any complement regulatory protein expression you can get in a transgenic organ. Under conditions, however, that dramatically block that response -- and hopefully these will be the same conditions established in the Gal transferase knockouts -- we see prolonged xenograft survival. So we think now that the current barriers to xenograft efficacy is perhaps a combination of an induced non-Gal immune response. Certainly it lies in the management of drug toxicity and infection in these pig-to-primate transplants. And finally, there may also be some still hidden physiological incompatibilities that need to be discovered and addressed. Thank you.

DR. SALOMON: With the permission of the Chair and Mary, I would like to just go right on to the discussion and then do the break. Would that be okay, Mary?

DR. GROESCH: Sure.

DR. SALOMON: Harold?

DR. VANDERPOOL: Yes.

DR. SALOMON: So while everybody's gathering their thoughts, I had a couple of questions. One would be to Julia and Randall. The way you guys have done this, you've got a neogene in your animal now. The current thinking is that neo will be an immunogene in humans. So haven't you created a problem for yourself? I'm sorry. That wasn't intended to ambush you or anything. That just came out in the last year. There's been a couple of papers on primates with looking at retroviral vectors, and there is some evidence suggesting that neo, and then there's some mouse studies suggesting EGFP as an immunogene, though those have been a little more controversial. I think the neo data is pretty good. And again I apologize.

DR. GREENSTEIN: That's fine. We chose neo because it was the best selectable marker that we knew of to generate these cell lines. One of the advantages of our tolerance induction paradigm is that we have the potential to induce tolerance to pig as well as neo if the pig thymus or the pig hematopoietic cells also contain the same genetic modification. Another approach is to try to reengineer the vector so that we can get rid of the neo, but that raises the burden on the molecular biology and the efficacy much higher and could be a second generation approach.

DR. SYKES: I have a question for Dr. Byrne. You showed us a very prolonged breeding scheme that would be required for crossing the Gal knockout pig to a complement regulatory protein transgenic pig, but in the mouse you can insert multiple transgenes together to make a transgenic mouse. I'm wondering wouldn't the quickest way to make a multiply transgenic Gal knockout pig or your ideal pig be to take the

homozygous Gal knockout pig once it exists and simply do standard microinjections of multiple transgenes into a fertilized egg from those pigs?

DR. BYRNE: Well, that approach may help you in terms of time, but I think if you look on paper, it might actually take just as long. Given just a male and female Gal transferase heterozygotes, it would take about a year and a half just to get a single Gal knockout litter on the ground. You'll be fundamentally limited by the availability of females in that breeding scheme. Then you need to start to generate a smaller herd of animals to create eggs for microinjection. The microinjection process itself takes a year and a half in order to create an animal. So you're getting to be two and a half, three years down the road there. I think it's just going to take a while to integrate. By classic breeding methods it's going to take a long time to put this together. Now, Dr. Greenstein illustrated that you could do what I describe as turning embryos. That is if you have an embryo that's a heterozygous for Gal transferase, you could take those cells out, then transfect them again, get the homozygous knockout, and for that matter, you could then take them out yet again and transfect in a piece of DNA and get a homozygous knockout with complement regulatory activity. Nobody's done that yet, so there's a fair amount of scientific question as to whether that's a doable prospect.

DR. SYKES: Still I really wanted to make the point that once we have a homozygous knockout pig, I mean granted that will take time in itself, but that animal could really serve as a platform for a multiply transgenic close to ideal pig in a relatively short period of time compared to the time it's taken us to get where we are now. I mean I think yes, it's going to be a few years, but it's not that long in the scheme of things.

DR. BYRNE: Well, a few years seems like a long time to some of us. I think the homozygous knockout pig, even in and of itself without any other genes, is an important animal to make and an important animal to transplant as soon as possible to see what effect it will have in terms of the immune response to that organ.

DR. SALOMON: I had a question. When you said you made 120, I think it was, different lines -- that was 200 -- I wasn't quite sure, but it's not important -- were those 200 or 120 different genes, and then you checked them all out, and ten of them gave you functional advantage, or were you looking at lines that differed, for example, by their expression or the mosaicism and things like that?

DR. BYRNE: Nextran has been making transgenic pigs for quite some time, initially as a company called DNX, and then later on after reorganization, as a company called Nextran. We've made animals with growth hormone genes. We've made a lot of animals with hemoglobin genes. We have tried all three CRP genes, DAF, CD59, MCP, as many genes as cDNA constructs and as genomic constructs, and in multiple variations of that scheme. So we've tried quite a few different construct designs, and at each iteration of that process, tried to raise the bar on the level of expression and the authenticity of expression to get an animal that we're currently quite pleased with.

DR. SALOMON: And I don't suppose any of that information is at your website. It's a joke. Next question I had was kind of going back to where Megan was going. That is let's examine the hypothesis that the Gal knockout is the base for the ideal pig. Guerard suggested an alternative perhaps by using what you called NEX 1285 as another way, but still I think we'd agree that if you really had a Gal knockout, that was the ideal pig. So the questions I had for all three of you is we know we have Gal negative mice, the Gal knockout mice. Are they totally normal? What's the natural function of the sugar? If you don't have Gal, you don't have the galactosyltransferase, do you just fucosyltransferase? Do you just make a different sugar, and is that going to be ultimately equally problematic?

DR. GREENSTEIN: I think going first to the mouse, as far as I know there are two independent lines

that were generated. One of them has been reported to have cataracts, and the other has variably been reported to either have or have not expressed cataracts. We don't know whether that's a function of the way the vector went in or whether it's really due to the lack of the enzyme. Other than that, they appear to be perfectly normal animals. So we expect that the pigs will be normal, but again until we have them, we won't know. In terms of looking at other functions, there's no model that we have except for the ABO blood groups where we know that humans terminally modify sugars on their glycoproteins in different ways. At least I think we would argue that an A person is as normal as an O person. I think it's nearly impossible to predict what the outcome of this modification is going to be until we have the animals on the ground.

DR. SALOMON: How about the last question. And that is do you have any sense of what will happen, maybe even from the mouse, that won't you just have a Fucosylated instead of a Galactosyl residue. It will be a fucosyl 1,3 or something like that?

DR. GREENSTEIN: That's right.

DR. SALOMON: And the end result of that will be it's not the ideal animal because you'll essentially develop an antibody response? I understand you won't have natural antibodies.

DR. GREENSTEIN: Right. So that's where you get back to control. I mean we're not predicting that this single modification is going to make the animal not recognized by the human immune system. We at least have very separate approaches to dealing with the subsequent immune response to the pig, and I think you'll have to have those approaches or at least very good immunosuppressive drugs.

DR. DALMASSO: Actually in the context of your question, the solution for that problem, if for example, other antibodies appear because the glycoproteins are encapped with alpha-Gal, the solution would be to prepare alpha-Gal homozygous knockouts and then prepare a transgene with human Fucosyltransferase. That will convert that animal into like a human blood group O with terminal Fucosyl.

DR. SALOMON: Interesting comment. Bill?

DR. SCHECKLER: A follow-up on that. It sounds to me like as you try to add a new gene or a different gene or subtract a gene, you're amplifying the possibility of unintended consequences in the animal that you wind up with so that you might wind up with something that will be more dangerous to use as a transplant source than something that is less dangerous or more manageable to use as a transplant source. Do I have that right? You said you really don't know even what the Gal knockout homozygous will be like until you have it, whether you'll have more than cataracts, whether you'll have septal defects in the heart or something else. That's the first question. The second question is you mentioned, Julia, that you were looking for PERV in all of the lines that you had, and you had some that didn't transfer to humans very well. I wonder in both groups whether, in addition to PERV, you've looked at other types of potential pathogens, since that's one of the major barriers for xenotransplantation from the public's point of view, not necessarily from the scientific point of view. But it would seem to me that it would be necessary to look as you develop these animals as early as you can for potential infections.

DR. GREENSTEIN: I guess to the first question, I think there's more technological risk when you're knocking out a homologous gene to have unintended consequences than perhaps there is when you're looking at dominant transgenesis. So for example, the addition of the complement regulatory genes, I don't think there's been any evidence that any of those pigs -- and there's probably a very large number of founder lines when you look across all the efforts. There hasn't been any consequence of any of those genes being expressed in the pig. We're much more concerned about it when we're trying to get rid of both alleles of a native gene. Hopefully we won't have to knock out any other genes in the pig to get to at

least some clinically relevant survival times. In terms of the second question, the vast majority of other infectious agents that we worry about are exogenous or not part of the genome, so we've tried to characterize at least our herd in terms of the endogenous viruses as best we can. We've participated in studies looking at CMV and how we can get rid of CMV in our herd, but until we have clinically realistic survival times, putting these animals into barrier facilities where we can get rid of the viruses or bacteria that are not part of the genome is a very costly approach to the problem. So we've in separate experiments proven to ourselves that we will be able to get rid of those potential pathogens, but we don't carry our lines that way currently.

DR. BYRNE: I would concur with the second part of that question. We have also populated SPF facility by cesarean derivation and have worked with it for a long enough period of time and bred animals within it to get a very good understanding of what sort of exogenous pathogens we can control. And other than PERV, which is clearly going to be endogenous to the pig, and some of the slower herpes viruses, the pathogens associated with the agricultural production of pigs really can be easily maintained. In terms of making changes in the genome, I think either adding genes or subtracting genes is always a somewhat risky business, and that's why I think it's very important to characterize the lines as thoroughly as possible using as many techniques as possible so that you can understand what it is you're working with.

DR. SYKES: I have two questions. First back to the theoretical risks of putting in antibiotic resistance genes to make the knockout animals. Is there any danger that that gene could be picked up by a virus and then transmitted to bacteria, thereby rendering bacteria antibiotic resistant in such recipients? Is that a feasible scenario?

DR. SALOMON: I don't think so.

DR. BYRNE: I'm not certain about the scenario, but the neomycin resistance gene is derived from bacteria in the first place, so it's not like they're acquiring a capability that they haven't already evolved.

DR. ALLAN: You'd have to argue that you could also transfer other mammalian genes into bacteria with the same process, and as far as we know it doesn't usually happen.

DR. SYKES: Thank you for clearing that for my mind. The next question is you have used cell lines from either embryos or very young pigs in the generation of these nuclear transfer donors. Is that because you want to avoid the consequences of telomere shortening and so on associated with an older donor or is it for a more practical reason?

DR. PRATHER: The telomere shortening question is a good question because apparently it's something that occurs in sheep but does not occur in cattle. In fact, the opposite occurs in cattle. The telomeres of these cloned animals are actually longer. We have sent samples to Jerry Yang at University of Connecticut, and he's evaluating them right now to find out what happened to the telomeres on our pigs. I'd like to make a point of clarification because somebody makes a misstatement, and then it continues. There are no septal defects in these pigs. Okay? So we haven't observed a septal defect. Ventricular problems, yes. Septal defect, no.

DR. SCHECKLER: I was just speculating about some unexpected consequence, not picking up from what Michael said.

DR. PRATHER: I know. And that kind of scared me because people will pick that up and carry that on and carry on misinformation. I just wanted to clarify that.

DR. SCHECKLER: Okay. Have anephric pigs then.

DR. SALOMON: Harold and then Jon.

DR. VANDERPOOL: Speaking as merely an ordinary American citizen, I think it's wonderful to be right here listening to the people who made such news just a couple of weeks ago. That's really neat. It is really good to have you personally here. My question to tie these sessions in with what we've been doing all day long is as follows. I'd like to hear other scientists come in and make comments. Let's assume that the excitement of the announced knockout pig included the excitement of a full knockout pig and that we actually have them here, and they're old enough for some transplants, and they're no serious genetic abnormalities that for whatever reasons could have occurred. The question I have is how far away would we be, in the light of all the other earlier presentations, from saying that we're ready to proceed with transplants? To what degree would we have to look at some of the other issues involving tolerance that we would have to deal with issues involving other possible carbohydrate secondary rejection mechanisms? My point is to keep these two things together, how far would we be? It seems to me that the state of the science group might consider that scenario as a possibility in terms of how fast and how far we've gone and how much further we need to go.

DR. SALOMON: Jon and then Louisa I believe. I'm sorry. I was thinking that was a comment, not a question. Well, I mean you guys can take me up on that. I personally think that's something we need to talk about tomorrow. I don't know how we're going to answer that now.

DR. VANDERPOOL: Okay. But my point is let's assume the promise is fulfillment here. How much in terms of the earlier presentations -- maybe your group needs to talk about this tomorrow, but I'd like to hear it voiced in the committee also -- in terms of your presentations earlier in the day would you still feel that a number of things would need to be done in order to be ready to move to clinical xenotransplantation?

DR. SYKES: I'd like to take the opportunity to partially answer that, and then maybe Julia will have something to add. In the two tolerance approaches that I mentioned this morning, the thymic transplantation and hematopoietic chimerism, studies in our research center in collaboration with Immerge involve a pig-to-primate application of those approaches. In both of those models the major limitation right now, or one major limitation to replicating what we have achieved in the mouse, is the murine models involve initial T cell depletion of the recipient to avoid initial rejection of the graft. We haven't yet had a reagent available to be able to do that in the primate. We are very hopeful that such a reagent is very soon in the pipeline, so that's not so far in the distant future. If that were achieved, then I would say that the thymic transplantation model in combination with the Gal knockout pig would be a very promising approach since it would induce T cell tolerance, and the Gal would be absent from the donor organ. We're hopeful that that might be the quickest approach to achieving prolonged xenograft survival. I don't know if it will be sufficient. The mixed chimerism approach, which I think has more barriers to overcome, that probably will take a few more years before we completely overcome them in large animals. That approach has potential to tolerize for other specificities once Gal is gone if those other specificities are important, and potentially to tolerize the NK cells and maybe other cells as well. So the answer from my perspective is we don't know, but once we have that double knockout, that homozygous knockout pig, we'll have something very exciting to try in combination with an existing strategy for T cell tolerance.

DR. SALOMON: If we're putting it into context, then I think it's fair to point out that the message I got from this morning was that that's not going to work and that the prothrombotic, procomplement activating quality of the porcine endothelial surface will continue to be a major barrier, and that will not be solved by a thymic transplant or a tolerance. That's just taking what we've learned today, Harold, and trying to answer your question. Does anyone else have a question? Eda?

DR. BLOOM: I think it's important that we also remember the infectious disease issues, and I know that today has not been -- sorry. I think that today, I know that today has not focused on those, but they're still going to be there, and those are still things we need to address. For example, is a Gal knockout pig more or less likely to produce PERV that is going to be more or less of a problem in humans? maybe you can make a Gal knockout pig on the background of these pigs that don't produce humanotropic PERV, and then you don't have to worry about it, but these are questions that still remain to be asked, and will need to be addressed before trials begin on it.

DR. SALOMON: That's a good comment. John, did you want to ask a question?

DR. ALLAN: Yeah, if I can talk. I don't know if I can talk --

DR. SALOMON: That's a rare pleasure, John.

DR. ALLAN: My voice is shot. But anyway, yeah, I mean I think that what you just said about, I mean the clinical trial thing is important to sort of always bring that up, but I think that the data is going to have to speak for itself, and I think that, you know, I mean the wonderful thing about the knockouts is your ability to put it into primates and see what happens after hyperacute rejection. I think that is just beautiful. I think you just have to wait and see what happens with it. But the second thing is is you can also deal with the infectious disease risk, at least in the primate model, because maybe you'll get longer survival. Right now, I mean you can only guesstimate with primates because you have such short survivals with a lot of this stuff that when you start getting longer survivals, you'll be able to start maybe getting some information that says, yeah, these are pretty safe, or maybe there is a virus there or something else. So I think it's, you know, you just sort of have to wait till it plays itself out.

The question I wanted to ask you, Julia, is and I may have missed this, is that in your -- in your pig knockout, did you actually -- did you look at surface expression? And did you look in different tissues as compared to wild type?

DR. GREENSTEIN: We haven't, actually, because when we looked -- when we did the first work with our vector, we looked at a stable cell line, and we saw no change in the expression level of Gal on the surface of those stabily knocked out 1 allele cells. So we had no reason to assume there would be a change in these pigs. And when the first one died post a blood draw, we really didn't want to go anywhere near them with any more syringes.

DR. SALOMON: Louisa, did you have a comment?

DR. CHAPMAN: Yeah, I -- and it's sort of the back side of Dr. Vanderpool's question, actually. I was going to try to summarize my understanding of what I think you've told us, and see if it's on target. And I think it's sort of another -- Dr. Vanderpool had said in light of what we just heard, and what we heard this morning, how much further do we have to go to get where you want to be? And I think my approach was kind of starting backing up, like how far have these transgenic techniques taken us?

So if I understood what you all were saying, most of the work that has been done so far with transgenic pigs have been done with three separate lines of transgenic pigs, but what they all have in common is that they're all pigs whole and complete in and of themselves that have had human genes added in, and three different kinds of combinations, but each one of those combinations was targeted towards the same thing, which was toward regulation of the human complement system, right? Was I right if I understood that right?

DR. BYRNE: (Nods.)

DR. CHAPMAN: So each one of those kinds of transgenic pigs was intended to decrease the problem Dr. Dalmasso told us about this morning, right, and then if I further understood you, each of them seems to have some beneficial effect. But that effect is small compared to the problem created by these -- by the presence of alpha-Gal, by the presence of this sugar on pig cells that isn't present in humans, which is a problem that Dr. Paulson was talking about, or a variant on it. But when, in animal trials, when things were done to control for that, to manipulate that to try to take it away as a problem, these transgenic pigs, with -- that had genes added to regulate the human complement system did seem to have a beneficial effect in prolonging survival longer than in organs from normal pigs, right? Okay. So now there are new methodologies for creating these transgenic pigs, and using those methodologies, someone has managed to create a knockout pig, where instead of adding in a human gene, what you've done is take away a pig gene, and that pig gene is the one that puts this sugar alpha-Gal on the pig cells, right? So you only halfway, you only have pigs that have this taken away from half their gene pool, right? But once you get them bread, in a couple generations presumably you'll have pigs that have had on neither half of their genes. So then we'll have pigs that, like humans, don't produce this sugar on their cells, so this whole problem supposedly of antibodies against this sugar that Dr. Paulson talked about should be eliminated. And then in that --

DR. SALOMON: No.

DR. CHAPMAN: Well, optimistically, that is the hope, right?

DR. SALOMON: You were doing fine, but leave Dr. Paulson out, because he wasn't talking about antibodies, he was talking about lectins, and it's got nothing to do with this.

DR. KASLOW: Those are different sugars.

DR. CHAPMAN: Okay. So one problem will be eliminated, that appears to be a major problem, or at least people hope it will be. And then in that setting you'll be able to see how far these other transgenic pig techniques have taken us towards minimizing the problem of complement activation that Dr. Dalmasso talks about, right? And then that will, to some extent, presumably affect the coagulation problems that Dr. Robson talked about, because some of those were kicked off by complement. But by and large, the problems Robson talked about are a whole 'nother kettle of fish that are going to have to be addressd in a different way. And that is true of every other speaker this morning other than Dalmasso, right? So is that helpful?

MS. SHAPIRO: Yes.

DR. VANDERPOOL: You've educated us several times today, Louisa, and this is one of those very good times.

You know, don't let Dr. Salomon's specific comments get in the way of a very good summary for many of us.

DR. SALOMON: I wasn't trying to get in the way. I was just trying to make sure that we stayed on track with that one.

I think, you know, just one person that we haven't heard from, and one sort of part of the story that we haven't heard from is just how people from the animal side view this, and Allan, I just -- I know I'm putting you on the spot, but I think, I don't think this discussion is complete, talking about transgenesis

and all this without some comment from you.

MR. BERGER: I don't think we've probably got the time, or if there are specifics that you want to get into. I mean there is -- I've certainly never hidden the fact that I am certainly biased against using animals in this particular way. I -- Let me frame it in a little larger scale. This being our fourth meeting, I -- for me personally, again, keeping my own bias aside, the most exciting thing that I've heard in our four meetings was really the artificial device side, which seemed to have the most promise and the less limits. I look at this, as we go through both torturing and killing a large number of animals, and we have problems from the immune response. We're having problems in terms of doing cloned pigs that we really may not necessarily know what will happen when we get to the end of that. We have infectious disease problems which are even a larger issue. And I brought up a number of times the use of resources, and how we have such a broad look rather than maybe focusing on something that may have more promise, and getting back to what I said, it just seems like, with a shotgun approach, we really use animals much more than we need to in ways that may not be very promising. And there are a large amount of people -- There is a larger part of the public that is really very sensitive to that.

DR. SALOMON: Fair enough. Are there any other questions or comments? Robyn.

MS. SHAPIRO: Following up on that, I just have one for you, for the two of you. It is still a little unclear to me what our charge is, actually, and in asking particularly the artificial device people that came to give a talk, is part of what you're thinking that we need to look at in the best interests of patients, reasonable alternatives, and that that may be one, and that our charge is to look at those two approaches, as well as -- I mean if we increased the human organs available through financial incentives, through presumed consent, through all sorts of things, I mean is that what you're thinking, is that we need to look at all of this in order to do our job here on xeno? Because I don't think all of us understand that, so putting this all in a context has been difficult.

DR. SYKES: Ah, I'm sorry, I just have to remind people of one comment that was made by Dr. Michler in his talk about the artificial heart, and that was that he thought in the end, a biological device was going to be the answer, not an artificial device.

MS. SHAPIRO: Right. I mean again my question was really to you two, to why you brought him here, to what your objective is for today, and all the speakers, and what you think our charge -- what we all think our charge is. I'm just unclear about it.

DR. SALOMON: I think you are clear. I mean I think you are clear about it. I think the point was said in the last meeting when we were talking about what it was you wanted from the science group, and that was that we weren't supposed to be cheerleaders for xenotransplantation. I don't think that is what the Secretary of State wants from -- I mean the Secretary of Health wants from us. And I don't know what Colin Powell wants from us, but certainly a solution to the Israeli problem, but I don't have one. But seriously, you know, I think that the point here was the charge that I made to the -- to the afternoon was essentially tell us what is going on, tell us what the competitive technologies were. And tell us whether if xenotransplantation is making enough progress, is it just should we just be patient, or is there -- is technology so compelling in other areas that maybe we shouldn't be going into all these controversial areas and driving, you know, off this edge of xenotransplantation, when resources and energy and focus should be put on emerging technologies that are better. Yeah, Bill.

DR. SCHECKLER: Just a follow-up from the other side of Robyn's question. And something else that we haven't really talked about, and that is reducing demand. Half of the liver transplants, if I understand things from Wisconsin correctly, are now done for hepatitis C, chronic liver disease, so a lot of investment in how to understand how to prevent hepatitis C would reduce the demand, at least in this

country, for liver disease. We just heard from our surgical colleague that congestive heart failure is the leading reason, and it has been rather flat as a reason for heart transplants. It's interesting to me that coronary artery disease has decreased substantially over the last 20 years or so, so reducing the demand would be a better understanding of all the thing that cause congestive heart failure. I don't know -- I doubt -- I don't know if Chlamydia is going to come up as the hepatitis C equivalent. I'd love it with my interest in infectious disease if that was the case, but it seems to me that that is the other side of the coin in looking at alternatives to any transplantation, that we haven't really explored, in that at least for the science group ought to at least be addressd in some sort of modest way as the other piece of this, if we are really going to be complete and circumspect in what we're advising.

DR. SALOMON: So I think what we're going to do now is take a break that I kind of put off, because I thought the discussion was really good, unless someone has some -- Allan, you had your hand up, I mean if it's --

MR. BERGER: Well, I was going to add, even throwing out even larger, I couldn't help but notice that -- that Julia's presentation talked about the diet that her own pigs got, which was a vegetable based diet, and when you're talking about prevention, which is what you're talking about, I couldn't help but note you were doing that to lessen the effect of disease, and I just wondered why we wouldn't be putting that message out there that prevention might be diet related that would also reduce that need.

DR. GREENSTEIN: Could I just clarify that comment? Those were directed toward infectious disease concerns that come from animal based feeds, not to the health of the pigs in terms of their need for further transplant.

DR. SALOMON: Okay, how about a 10-minute break, and then we'll finish up.

(Break)

DR. SALOMON: We can get started on the last part of the afternoon, almost evening.

DR. SYKES: Okay, so our second-to-last speaker, our penultimate speaker for the day is Dan Salomon, who is going to tell us about cellular xenotransplantation.

DR. SALOMON: Just to show you that revenge comes in many ways, there is a -- Louisa put a -- a present for me on my computer here called Sweet Dreams Herb Tea, which she is encouraging all audience members to try at the beginning of my talk. Thank you, Louisa.

Anyway, I'm going to try and briefly review a little bit about where we're at in cellular xenotransplantation, see if this wakes up here. When I did a -- a literature search on this, while this is waking up, there were 2,500 plus abstracts pulled up on Medline OVID under xenotransplantation cell. And then if you put xenotransplantation cell clinical, it dropped to 456 abstracts, of which 450 were reviews and discussions, and which I have to say I have gone to a couple of them, and then there were six, I pulled six references that were specifically on cell transplantation. So, let's just kind of go, put a larger context on it. What are candidates for xenogeneic cell transplants and tissue engineering? Obviously diabetes and islets, and the next talk is going to be on that, so I'm going to try and skirt around that issue a little bit, but there is something compelling we'll have to get into. Certainly liver failure, hepatocytes, either loaded into devices as we already heard in great detail from Gary Levy, and cell transplants, which Gary also mentioned. Neurologic diseases I'm going to provide some more discussion of, because I think of this list, diabetes and neurologic diseases with neural cells, I think are the two most compelling candidates for the next series of gene trials, and that may not be. Everyone may not agree with that. But, muscle diseases with myocytes, let's not forget HIV immune deficiencies with hematopoietic stem cells,

or thymus, I think even though this has been tried once, it is really not in any way definitive, and I still think this is a potentially interesting area down the line. And then rejuvenation, really any cell that sounds logical. I didn't know whether this was ever going to get published. It initially read "Any Cells That Somebody Was Stupid Enough to Put in Somebody," but we'll get back to that.

How sound is the scientific rationale for xeno cell transplantation? First consider the rationale for cell transplantation. And the bottom line premise, from my view, is that many disease states require a dynamic, constant, complex, highly regulated, and long-term intervention. And that may forever elude simple drug and biological interventions. I think if you think about diabetes, I think there is no question about how powerful insulin is. If you think about Parkinson's disease, you've got antidopaminergic agents. I mean just think about any of several different disease processes, in the end it's some of these by -- The true nature of biological complexity is such that many of these diseases will really only be satisfied by a biological replacement.

Second, consider that fetal, neonatal and adult animals represent a renewable resource for cells critical to disease states. And also consider that transgenic engineering enhances the value of animal cells and tissues for these sort of applications. And I think that together, that provides some set of scientific rationale. I want to put this into context. Again, I thought Robyn's point was extremely valuable, that is if there is any confusion about where we're going, let's, you know, make sure we get it out. So from my own personal view, this is where I -- where I see this part, cell transplantation and tissue engineering go. In the first stage, what we're doing is we're transplanting essentially fully functional tissues, and our challenges are not to make these fully functional, because they already are, but rather to preserve function by engineering revascularization and engraftment in the host. And islet transplant would be a good example, or a heart transplant, for that matter, but that is not tissue engineering.

Stage two, we're doing something new, we're saying we're going to transplant stem cells, or maybe a little further down the lineage pathway with fetal progenitors, and then our challenge is not only engineering revascularization, but also proliferative expansion, differentiation along a desired lineage, and stable functional integrity. And that is a really big deal. But in the third stage, we're going to the last step, I believe, and that is actually using autologous adult stem cells, directing them to a desired site, regulating their differentiation and repair of a damaged tissue. This can be xeno, this can be xeno, this isn't xeno anymore.

Are the barriers to xeno cell transplants different than for whole organs? Yes and no. Yes, xeno cell transplants are vascularized by host vessels, Dr. Platt made that point this morning. Immunologic responses to cell grafts are different than the whole organs. Certainly a lot of these acute complement and thrombotic events may not be as critical for cell transplants as for organ transplants. But keep that thought when I show you some data from Carl Growth (phonetic) in a minute on islets and complement inhibitors.

Responses to fetal stem cells or other stem cells are unique. Early in differentiation pathways, expression of MHC molecules is very different than later. And so the early stem cells are -- are MHC lower, even MHC negative. And then as they differentiate initially upregulate Class 2 molecules and then Class 1 molecules. So this is a very interesting area to keep track of. And certainly engineering cells is easier than engineering whole organs, and engineering cells is a whole lot easier and faster than engineering whole pigs. But no. Xeno cells are destroyed by natural antibodies, just as the organs are. They produce infectious agents, just like the organs. They do not, at least to date, function any better in the clinical trials. I mean it would be great if I told you that, you know, they put a bunch of xeno cells in, and you're getting hundred percent cure rates in disease process. But that is not true, albeit perhaps for different reasons. And they have many of the same cell biological challenges to survival and function as whole organs.

Let me spend a couple minutes talking to you about neural stem cells, coming back to what I propose to you is that I think that these and islets are the two sort of foci, I believe, where the next steps in cell transplantation with xeno is going to go.

There certainly are a lot of serious neurodegenerative diseases in the CNS that are good targets for this sort of an approach. These are also diseases that have very little other therapies. We are facing an aging population, and these people are willing to participate in these sort of clinical trials, so I think that for all these reasons, these are potentially good targets for this sort of an approach.

Huntington's disease, Parkinson's disease, different forms of dementias, Alzheimer's potentially, are all possible targets for these so-called restorative or regenerative strategies. Just to give you a background of what has been done, they have done 12 patients with Huntington's disease, 12 patients with Parkinson's disease in Phase 1, it's our my honor, really, to know Jim as one of the people that participated in this initial phase. Eighteen patients then were done with Parkinson's in Phase 2. This was nine with fetal pig neural mesencephalon cells and nine not actually done in a controlled blind, I guess -- I guess it was blind, yeah, a controlled study, which is kind of remarkable. You got to give these guys credit for a very, very well designed trial with objective end points. However, this is an area with very difficult to control features, and even though they used the state of the art objective end points, that is still also, you know, quite a difficult thing. It's not like measuring the creatinine or hemoglobin. No objective benefit was demonstrated, but tell Jim that, you know. And Jim and I have talked about that. And it does explain, you know, that any of us who participated in different clinical trials that there can be unexpected individual variations, and there are issues of these difficult to control outcome parameters. So even something that appears in very well designed trials is not demonstrating benefit. One has to keep that into context.

The potential of neural stem cells in the next two slides, I believe, three slides, coming from John Paul Sulu's (phonetic) program in Nantes. I sort of sent out feelers to a number of people in the area to send me PowerPoint slides so that I could make sure I was as up-to-date as possible. So John Paul and his group argue that they're easy to obtain, they're self-renewing, they have a low immunogenicity, they're multipotent, they can give rise to all three main cell types in the CNS, and they're transfectable, so they're targets for gene therapy. I'll show you an example of that. The limitation is that they do seem to preferentially differentiate into astrocytes, and that may be something that they can get around if they understand more about the genetic program and some of the signals so that that can be manipulated. There are a large amount of neurons that are difficult to obtain. It's difficult to obtain a large number of neurons, and some neuronal phenotypes are difficult to obtain. And this is sort of a picture that he sent me of where their program is going, so they're going to embryonic brain, pulling out these NSC neural stem cells. These are multi-potent self-renewing. They can go down several different pathways into the different types of cell types, so you got glioblasts, astrocytes and then neurons. And I mean again I don't want to get caught up on the details, but he is pointing out that these neuroblasts, they're gabanergic, and dopaminergic and glutaminergic and cholinergic and others, and these would be suitable for therapeutic applications and a whole series of things, including neurodegenerative diseases, trauma, ischemia, stroke, multiple sclerosis, et cetera.

Now another group in the Nantes group under John Paul Sulu is doing something else that is kind of putting together, I think, both neural stem cells and transgenic engineering. So what they did here was they made a transgenic pig that had a vector in which the on switch, the promoter, is a neural specific enolase gene, which means that only the neural cells express the payload gene of the vector. So that switches it on, the payload gene is transcribed, and it makes, in this case, CTLA4Ig, which is an immunosuppressive biologic.

Their idea now is you are going to take these transgenic pigs as the source of fetal neural stem cells, or adult neural stem cells, and inject these in the brain. And they have been successful in having eight transgenic strains that express this soluble immunosuppressive biologic in situ. Just I think we're allowed in California to say cool once a day. And I think this is cool.

Pig islets for diabetes, again, I think we're going to talk about this next, so I just want to kind of just again put a context here, these have been encapsulated in alginate capsules. They have been given by portal venous injection, that has been the majority, and of course right now the tremendous excitement over the Edmonton protocol, and other new advances in islet transplantation have pointed to this portal venous injection route as the current favorite.

Carl Growth has put several -- done several patients under full immunosuppression under the kidney capsule. One of the ironies has been that in the mouse model, putting islets, or in the rat model, putting islets under the kidney capsule is the gold standard of this sort of murine small model. But in kidney transplants, you basically remove the capsule and devascularize it. So no one was very interested. The transplant surgeons weren't interested, because it's absolutely the wrong site for a kidney transplant.

And interestingly only Carl, to my knowledge, but Brad you might correct me, has done in a native kidney a subcapsular kidney transplant -- islet transplant, which I thought was really very interesting. And again, these -- They've -- a couple of them I'll come back to it, but this has not cured anybody's diabetes, and we'll get back to that in a minute.

Gal or not to Gal, there have been discussions that islets don't express Gal, then everybody looked at the data a little more carefully, and islets express Gal. So I think it's very interesting to look at the fact that the Gal knockout mouse is going to be -- I mean pig is going to be as interesting to cell transplantation potentially as to organ transplantation -- vascularized organ transplantation. And I want to show you something from Carl Growth. So now Carl sent me sort of updating me on what he was doing. Sorry. My computer decided to go to sleep. That is not a hint for any of you. Intraportal injection of porcine islets to cynomolgus monkeys treated with this soluble complement receptor 1 inhibitor called TP10, and what you see here on the vertical axis is soluble activated, what they call MAC, or membrane attack complex. So it's a marker, so the higher the soluble C59 is in the serum, is a marker of -- or plasma, rather, is a marker of complement activation. So in the animal controls, you see a very clear, this is on days, the islet transplantation is done at day zero. You can see the very active complement activation. So one of the points here, which is interesting getting back to this morning's sessions, is that even with a cell transplant, regardless of this argument that the cell transplant is going to be vascularized by host vessels, and therefore we don't have to worry about these things, I think this data would suggest that we do have to worry about these things.

Of course he put it into the intraportal main, so it was immediately in contact with blood. It is possible that one could argue that if you put it into a tissue compartment where you didn't have immediate contact to blood, this may not be a factor, but then the question is what tissue compartment doesn't have any blood. So I think you go around in circles, and this is still a problem. What is very exciting about this data is that when he exposed the islets to this complement receptor blocker, and -- I'm sorry, and gave it then to the animals, up to a week you can see that you had no measurable activation of the circulating membrane attack complex.

And this is -- This is a second slide he sent me, which is a -- looking in the liver, this is a pig islet that is without the complement inhibitor, and this is the pig islet with, and you can see here that this is surrounded by a -- a dense lymphocytic infiltrate. And it's really pretty torn to shreds, and this one looked perfectly happy sitting in the portal triad, and this is a very typical sort of structure for a pig islet. One of the things that is troubling with pig islets, if you're working with them, is that they don't have these really

nice, neat balls. They fall apart rather easily. So this is really a beautiful example of a pig islet in situ.

You can't always pick your timing. I know the charge for today was science. I will do this briefly. But the next two slides, I think, are very controversial, and I want to be very careful. The way I know about this is through three ways, and this has to be up front. The first way is through the press, the second way is Sir Roy Kahn (phonetic), who many of you know was a visiting professor at Scripps at my invitation about a month ago. He had just come from Mexico City, where he had examined these patients. He had -- He trained the guy at Children's Hospital who is doing these. And the third is personal interaction with David White two weeks ago, and David is one of the collaborators on this project in London, Ontario. So please take everything I say now as in that context. It is verbal from good people. I have otherwise no vested interest in any of this. But I just wanted to make sure that that was up front with everybody.

So what we understand is that a clinical trial is presently under way in Mexico City, it's supported by a New Zealand company called Diatranz, and it's reportedly approved by the Mexican government. The candidates are again, I'm told, children with difficult to manage diabetes. I'm told that is particularly difficult to manage in Mexico. It's a device that is a -- essentially a metal device that they implant in the rectus sheath in the abdomen, that is the two large muscles that form the four-pack, or the six-pack that, you know, all the young guys want when they're weightlifting. And there is a trocar in it, and then they come back several weeks to several months later in a second surgical approach, pull the trocar out, which creates now a space, and they put into the space a -- a -- the islets in a mixed -- these are neonatal pig islets that are made in New Zealand that are mixed with a cell called the Sertoli cell. The Sertoli cells are in the testes, and they are known to express a lot of phos and possibly also make a number of immunosuppressive thromboxane derivatives, the exact reasons are not clear, but they are immunosuppressive. And this combination of Sertoli cells and neonatal islets, all porcine, have given at least two children complete insulin-free existence, according to what I've been told, and Sir Roy reassured me that he actually examined these kids. And there is a third child who is of 12 who has a significantly reduced insulin requirement. Again, this has not been published, and I think I've been straight about where I got this. There is no immunosuppression, from what I understand, and reportedly the neonatal islets, though I don't know anything about the Sertoli cells, do not make infectious PERV, which of course I don't believe. I have published, and others have published that islets -- pig islets, fetal and adult, make PERV and are infectious, makes productive infection, but you never know. I mean they might have a DD variety that is not hemotropic it's not for me to say, not having seen the data, and that the patients are being monitored by international experts.

Well, why quit when you're on a roll. The same company has recently announced its intention to do a similar trial, though I have no idea of the details in the Cook Islands. The International Xenotransplantation Society sent a warning letter to the Minister of Health in New Zealand. There will be editorials in "Transplantation" and "Xenotransplantation" shortly on this. It was noted in the press that when a caution was raised by the health minister in New Zealand, one of the politicians in the Cook Islands protested this unwarranted interference in their sovereign affairs.

So, let's bring this kind of down to the end here. What is the level of success presently documented for xeno cell transplants? Well, pig islets secrete C peptide in human transplants, sometimes quite long-term, months. There have been, however, little or no sustained change in insulin requirements. Baboon hematopoietic stem cell transplant to the HIV patient did not engraft. I believe we were in this very same room when we proved that. And there, to my knowledge, have been no repeats of this. Fetal pig neural cells have had no demonstrable benefit. But I think I've made my comments clear about that. There may be individual exceptions.

Neonatal pig islets in children, I just reviewed that, there may have been a cure, that is pretty amazing.

No data has been published on shark neural cell transplants done in Mexico for spinal cord injuries. This is the beginning of my sort of xenotourism argument. At the beginning I know you guys are probably tired of that, but I don't think it's going to go away, and I hope we can have some discussion at some point on these things. And no data has been published for a whole series of ongoing administration of xenogeneic cells for rejuvenation being done in several places, in both Mexico and Europe.

Okay, what is the competition for xeno cell transplants? The primary competition is a variety of human stem cell strategies, embryonic stem cells, fetal stem cells, and adult stem cells, and the point here is, is that one has to take stock, and I think we've already done that today, you know where is stem cell research, where is xenotransplantation research? And I see no indication that stem cell research is advancing so rapidly and in such a promising way that it casts too large a shadow across xenotransplantation. But is it a competitor? Absolutely, I mean we've got to put that in the mix.

Secondly, gene delivery to create novel surrogate cells. You know this, I think, is really amazing, there was some -- There was recently evidence that, and this is just, I think, very intelligent work, so they -- we know we can put an insulin gene in, but the trouble is, is that everything else upstream of it isn't there, so glucose sensing and release, and the whole thing, so what these guys did was they found these cells in the small intestine that are a type of stem cell, and they sense glucose, too, interestingly enough. And that is not so crazy, by the way, because there is increasing data now that the pancreatic bud and the intestinal gut stem cell is actually one in the same. So the fact that there are intestinal stem cells doing something similar to what pancreas endocrine cells are doing is actually not that great a leap, in terms of now our growing understanding of embryonic stem cells. And they -- These cells sense glucose and secrete a gut peptide that may have something to do with a glucose response loop in homeostasis, but a minor one. So they stuck the insulin gene on the upstream of the same promoter and cured a bunch of diabetic animals. That's a very neat way, I'd say cool, but I'm from California. I can't say it again. But it's a very neat idea of, you know, how to use, you know, growing molecular understandinging of stem cells to engineer surrogates, beta cell surrogates.

Devices are still possible for some applications. I mean if you could come come up tomorrow with a glucose sensing insulin pump, this has not been an easy objective, but it's not impossible with some of the new biosensor technologies that are being tested. And certainly hepatic assist devices are out there. Then I think we can't ever forget that the continued enhancement of drug-based therapies always have to be considered. In other words, still, even though I might argue that this dynamic and complex disease processes don't always lend themselves to simple single drug therapies, the fact still remains that if you got a tremendous benefit from drugs, we know some of these drugs are really very useful, you'd still have to put that into context in looking at risk/benefit ratios for xenotransplantation.

So this is my last slide. Why doesn't xeno cell transplantation work now? What is -- What do we have to do? Well, essentially in my opinion the same set of challenges face xeno and allo cell transplantation. That is in some sense kind of encouraging, because it means that the advancements in cell transplantation with xenogeneic cells as well as adult and fetal and autologous human cells and allo cells will march together in progress. That is a good thing. So those challenges include cell harvesting, ex vivo culture and manipulation, revascularization engraftment strategies. What is the best cell choice for cell transplantation? Again, should they be mature cells, like do you pick mature islets, or do you look at lineage committed or various kinds of stem cells? This is a fundamental question, again, that is not just for xeno.

One of the interesting questions would be so you get a stem cell to make it into a beta cell, and now you think you've got an insulin-producing beta cell surrogate. Well, you do. But the islet has got glucagon producing cells, pancreatic polypeptide producing cells, somatostatin producing cells, great, now you've got this pot of beta cells. Is that really enough? Or is the islet smarter than us yet? And is it just a little

bit of us being arrogant and saying, "Oh, yeah, we'll just put in insulin and we'll control it any better than insulin injections."

Additional barriers, I think I have been fairly clear about. And I just want to leave you with, this is a line that got dropped out of the movie Jerry Maguire, but it will be in the director's cut, that is "Everybody has told me about da money, now show me da benefit." I think that the major thing here is to say: One, whatever we do to move these technologies forward, it's going to be based on benefit. And I think that is a theme that has come up several times this morning. And in that context, one of the issues that has been raised with these trials being done or proposed outside the, you know, in Mexico and in the Cook Islands, is where is the benefit? Thank you.

(Applause).

DR. SALOMON: I think I'll sit down, and we'll do the second talk, unless there is some point of clarification.

DR. SYKES: Dr. Zhong?

DR. ZHONG: Excellent talk. I would like to add a few about the Mexico experience, because, first of all, the abstract about this work has been published in the June of Xenotransplantation. The -- Because this group report, they are printing in the last year International Xenotransplant Conference. I add a few detail, because my colleague, David White, actually did it all the immunology study for this group. The total is 12 patients, and all these patients had severe diabetes. Their C peptide was zero before the transplant, and the five of them had a significantly improved after transplant. The amount of insulin required for these five kids actually reduced more than 40 percent compared previous surgery. And three of them were off insulin. The longest one was more than one year. And also interestingly in these patients, the -- if you look at the antibody, they have a very high anti-Gal antibody, but for some reason, these -- the antibodies do not kill the islets. So accommodation may occur in these patients. So I understand from our ethical point of view, it is, you know, not quite right to start a clinical trial in Mexico, but we cannot stop them.

I would like to make a point, the islets transplant, xeno islet transplant, is extremely important. In Canada, you know, my colleague in animal, develop a beautiful allotransplant program, but the problem in Canada is every year we only have 400 pancreas, so let's say maximum we can do 200 islet transplant. Usually we need two pancreas for one recipient. But the number of diabetes in Canada, I think, are close to the one million. So allo islet transplants will never be a clinical option until we have pig islets.

The second thing I would like to emphasize is the islet transplant, cell transplant, Dan, I agree with you, is relatively easy compared to solid organ transplant. And also tolerance is relatively easy in islets. For example, the -- We -- Pig islets can be transplanted in the mice with a minimum immune suppression. We even do not need a bone marrow transplant. So for us, there is two things we would like to do: Number one, we would like repeating these experiments in the monkey; number two, we would like to develop a tolerance strategy in this model, because in the Mexico experience, they do not use any immunosuppressive drug. So there is three of 12, are effective. I was just thinking if we add some tolerance strategy, we may significantly improve the success rate. So personally I believe pig islets transplant is a very promising field we should really support this program. Thank you.

DR. SYKES: Well, while I agree in principle with the potential importance of pig islets, ah, in xenotransplantation -- I mean in transplantation, because there never will be enough allogeneic islets, I think there is some really serious concerns, as you have mentioned, particularly the -- the issue of doing a study abroad in a developing country. I would like to add that some information from a draft form of a letter that was sent by the IXA president at -- past president and future president, to the "Journal of

Xenotransplantation," and "Transplantation," in which they point out that the pig tissue that was used by Diatrans in these patients did not even have as little as current GMP certification, and that the trial had been refused by the New Zealand regulatory authorities, and the investigators were not permitted to carry out the study there because it did not meet regulatory requirements. So I think there are some really serious issues. And on top of that, as far as I'm aware, Sertoli cells in combination with islet transplantation, have only been shown to be beneficial in -- or to work in immunosuppressed rats. As far as I know there are no large data -- large animal data supporting this approach, even with immunosuppression, and absolutely no data to support its use without any immunosuppressive drugs. So in the absence of adequate animal studies to support the clinical trial, I think we have to consider this to be really, I would say, unconscionable, despite what beneficial results we may be hearing. I think that, you know, the -- this is the wrong way to go about xenotransplantation. I think there has to be good animal data to support clinical studies, or we're going to end up making a lot of mistakes.

DR. SALOMON: I agree completely. I think what -- Yeah, no, John, I'll call on you. I think what I would like to do, rather than stay on the science theme this evening, but as I said, one can't always control the timing of these things, and I personally didn't feel like I could leave it out of the talk, but, Harold, with your permission, I'd like to put it on the agenda tomorrow, since with the specific thing that I think this committee should discuss, the proposal that this is something the Secretary of Health should be apprised of, that this should go to the president, the president should call Vincente Fox, get together on one of his ranches somewhere, and end this.

DR. ALLAN: I'd second it.

DR. VANDERPOOL: I would certainly entertain that as an article for this committee to deal with, and we can each and all think about that issue -- over dinner. We're going to -- We may not get -- We may have to skip dinner if we keep going with this session. But I think that is a good thing to entertain.

We've talked about the need to deal with international issues, and this is one of those really hot spots that I don't think we can say, well, no, let's wait for our July meeting. I think we probably need to talk about this -- about this tomorrow, and I welcome that as an addition to tomorrow's already filled program, given the fact that we are supposed to leave by 2 p.m. tomorrow. Shall we proceed to the next speaker at this point?

DR. SALOMON: Right.

DR. VANDERPOOL: I think we certainly have our ears tweaked by what we've heard from three persons, Dr. Zhong and Salomon and Sykes. So I think we're prepared for some fireworks, however brief they may be, tomorrow.

DR. SALOMON: The last speaker of the day, and but certainly not the least, is a very patient Jim Wright, from Dalhousie University, Canada, and this is fishing for a cure, piscine or tilapia islet xenotransplantation. I understand that tilapia is also a delicacy in Thailand.

DR. WRIGHT: That's correct. So anyway, I'm the token non-pig xenotransplantation speaker. I'd like to start out with just a kind of a momentary summary of islet allotransplantation, and that is transplanting human islets to diabetic patients, and in Type I diabetes, the problem is that the immune system has destroyed the beta cells, which are the insulin producing cells in the pancreas. So although patients can be maintained with daily multiple insulin injections, over a period of time they develop a number of long-term complications. And so the idea behind islet allotransplantation is that it's a more physiological treatment, it should minimize the variation in blood sugar levels. The expectation is that this would prevent the complications of diabetes, but it's still in experimental treatment, and it's been very slow to

develop into a clinically useful modality, notwithstanding recent data from Edmonton. And there are a number of reasons why it's slow to develop into a clinical useful modality.

This is the microscopic appearance of the human pancreas. The islets, as shown here, are these pale islands of cells each representing two to 3,000 cells, represent about 1 percent of the pancreas. So it's very difficult to extract the islet tissue from the remainder of the pancreas. So one goes through this complicated islet isolation procedure, which is characterized by high cost, roughly about six thousand U.S. dollars per pancreas, low yield. It takes about two to three pancreases to do a transplant. The yield is also variable, probably 10 percent of all islet isolation procedures fail. But the major issue that we're all aware of is the shortage of human cadaveric donors, and in all of North America, there are roughly probably about a million to a million and-a-half Type I diabetic patients. In a given year, there are about five thousand potential -- five thousand donor pancreases available. So the supply and demand -- or supply will never reach the demand. There are a number of problems associated with islet xenotransplantation. I've listed a number of them here. The only one I'm going to really discuss today is number one, Selection of a Donor Species.

While most of the world interested in islet transplantation is focused on using pig islets as a donor source, the reason for this is that Porcine insulin is almost identical to human insulin, and there are actually a lot of physiological similarities between pigs and people. And for those two reasons, pig islets would seem to be a good source. Our laboratory has taken a kind of a radically different approach. We're using a large tropical fish called a tilapia, and as Dan alluded to, very popular in Chinatown everywhere.

And the -- Anyway, the reason for using a fish donor, is that fish have an -- at least certain types of teleost, or bony fish, have anatomical separation of the islet tissue. So it's a separate series of organs, and not part of the main pancreas. Therefore, it's very easy to extract the islet tissue.

This is a large tilapia islet and a smaller tilapia islet at the same magnification as a human pancreas. Pig pancreas looks roughly the same. Right here you can see me outlining a large human islet. So you can see that basically the tilapia islets are pure islet tissue, and they're massive relative to human or pig islets. So these, as I refer to BBs here, or Brockmann bodies, are these fish islets named after Brockmann, who was an ichthyologist in the 1840s. But anyway, these Brockmann bodies, very simple to harvest, but the first question was whether they would function if you put them into a diabetic mammalian recipient, and to do that, we used nude mice, which in addition to having no fur, have no T-cells, and therefore don't readily reject grafts.

The mice remained diabetic with streptozotocin, which destroys the beta cells in the native pancreas. As you can see here, this is the starting blood sugar level prior to transplantation, and then after transplantation into these diabetic nude mice. The islets were transplanted under the kidney capsule. You can see that blood sugar levels were maintained in a mammalian range for 50 days. At that point, the grafts were removed, and the blood sugar levels returned to a diabetic state. This slide shows just a histology of that. Here is the kidney, the kidney capsule, and you can see one of these grafts, fish islet grafts, under the kidney capsule. The same thing stained here by immunoperoxidase for insulin showing the remaining graft. However, when you take these fish islets and you transplant them into a mouse with a normal immune system, rather than a nude mouse, they reject in roughly seven to eight days. This is the same length of time as it takes for human islets, pig islets, dog islets, any kind of highly discordant islet to a mouse, basically you'll get rejection in this about this time frame. The rejection -- Here you can see histologically this is where the graft formerly was, and you can see that there is quite an inflammatory infiltrate that has attacked this.

This slide, basically, I'm not going to go through it in detail, but basically just shows that the components of the immune attack are eosinophils, microphages, and then later CD4 and CD8 positive T-cells.

So there are a number of different ways to prevent islet xenograft rejection. I'm really only going to talk about one of these immunoisolations, but I'll just digress for a moment related to immunosuppression. By and large, if you're transplanting islets across a wide species barrier, you can prevent rejection with very high levels of immunosuppression, but usually to the detriment of the recipient, so it's not an efficient way. The only exception to that in our model and in most models would be anti-CD4 treatment, which, if you can maintain that, seems to be highly effective.

Immunoalteration, immunoprivileged sites I'm not going to discuss at all, but I'll just tell you neither one really works in a discordant islet model.

Immunoisolation is the main area that most people that are looking at transplanting islets across a wide species barrier are interested in. And basically, the idea there is that the islets are encapsulated in very tiny microcapsules, which are generally made out of substances such as alginate. They have a porosity which allows small molecules like insulin, glucose and oxygen to move in and out freely, but larger molecules, like immunoglobulins, antibodies from the immune system, cells from the immune system, presumably can't get at the graft.

However, there are a number of problems that are shared by all types of islet encapsulation devices. First of all, none are entirely biocompatible, which means that there is always some kind of either fibrotic or immune response directed at the capsule material, particularly when there is an islet inside.

Graft hypoxia is a major problem, in that the graft, basically you've removed the blood supply from these islets, which normally would be a very vascularized tissue, and so because of that, oxygen has to diffuse through the device, then through the dead space into the device, and then into the islet tissue, you end up with a loss of islet cell over time. Total graft bulk increases, durability can be a problem. If you decrease the thickness of the capsule wall in order to maximize diffusion, then you can run into durability problems. There are all types of issues which I won't go into detail related to CHEF antigen, and the possibility, or the very definite possibility that cytokines can diffuse in through these small pores, and then all these devices, even under the best of circumstances, have to be replaced or refilled periodically. So what -- This is a definition that I like very much for what is the ideal islet for encapsulation, and it would have high insulin output correctly regulated by glucose and other secretogogues, low metabolic demand, capable of functioning for extended periods without replacement, and then must be procurable in high yield at reasonable cost with protocol meeting FDA standards. So that's quite the task.

I'd like to, now focusing on the fish islets again, as I mentioned before encapsulation causes real hypoxia problems, so low oxygen levels within the device for the islets. And the fish islets and fish cells in general are actually very suitable for this type of thing. If you consider that water contains very little oxygen, warm water contains almost no oxygen, and fish that can live in these kind of stagnant water conditions, obviously their tissues tolerate very little oxygen tensions. And this is an experiment we did a few years ago where we took fish islets, we broke them up so that they were the same size as mammalian islets, and then we took mammalian islets, we cultured both under as low oxygen tensions as we could generate, and under these conditions, at 24 hours you can see the fish islets still look nice and round and healthy. The mammalian islets have fragmented. This is fluoracine diacetate ethidium bromide (phonetic) staining. Each of these, as I'm circling, are islets, so these are two to three thousand cells. This is what freshly isolated mammalian islets look like with this stain. Green shows viability, orange shows cell death. And at 16 hours, the mammalian islets look like this, just a few clusters around the outside of viable cells. The rest is dead.

Ah, this is fish islets under the same conditions at seven days, so you can see that there is a marked difference in the level of viability, and we could actually transplant these fish islets and still show

function.

The encapsulation device that our lab uses, because the fish don't -- the fish islets don't really require the same level of oxygen, we use a very large diameter encapsulation device. These are 4.5 millimeters in diameter and we can get a lot of islet tissue into each one of them; whereas, the standard microencapsulation device for mammalian islets is in the range of around six hundred to nine hundred microns in diameter.

If you transplant these encapsulated fish islets into diabetic mice, you can get reasonable long-term survival. When you remove the graft at the end, the blood sugar levels come back up. In mice -- In nude mice, there was no need for any immunosuppression. In mice with a normal immune system, we had to give low dose immunosuppression to get good graft survival. This just shows one of these encapsulation devices. The size of this would be basically -- I'm outlining it here, but you can see the fragments of -- of fish islets inside. This is the the edge of the device right there, and this is a higher magnification stained for insulin. You can see clusters of beta cells, and then also non-beta cells still present as well.

So how do these tilapia islets compare with mammalian islets? The one general observation from the islet xenotransplantation literature is that pancreatic islets have species specific set points for normoglycemia, and therefore the islet grafts regulate glycemia in recipients according to the norms for the donor, rather than the recipient species. So in that context, this slide shows normoglycemia, both fasting and non-fasting for tilapia and man. You can see they are quite similar.

To get an idea of how these function in the set point, this is an experiment from a few years ago where we transplanted equal numbers of rat islets, mouse islets, or fish islets under the kidney capsule of diabetic nude mice, and you can see that the starting level, all three came down, and you can see that basically the rat and the fish were superimposable, whereas the mouse has a higher set point, and so had higher blood sugar levels.

At the end of 30 days, we performed glucose tolerance tests, where basically the mice for all three species were fasted overnight, then injected with glucose. Blood sugar levels go up, and then you can see that blood sugar levels came down quite rapidly.

The -- One of the issues related to xenotransplantation is, as Dan mentioned, there are other cells within the islets other than just the cells that make insulin, and so we did a study with Mike Conlon, where we basically purified and sequenced all of the different peptides that are produced by the tilapia islet. So we had an idea of what was being made, and how biologically similar it might be to mammalian -- to the various mammalian peptides.

If you look at islet cell composition in tilapia versus man, you can see that there is quite a difference in the different cell types, the beta cells which make insulin comprise 70 percent in man, only 42 percent in tilapia. The alpha cells that make glucagon 11.5 percent versus 20 percent. The somatostatin 14, which is the somatostatin that all mammals, or actually all vertebrates make, is identical. You can see that the percentage of these is higher in tilapia than in man. And then fish, in general, also make a second somatostatin product which is called a large somatostatin, which is unrelated. It's a different gene product, preprosomatostatin 2, which is not expressed in mammals. So we wondered whether there might be a problem having 23 percent of the cells making a product that would have no known biological activity in mammals, and might form immune complexes.

But -- Actually, this next slide just basically shows the topography of the islet cells. This is -- You can see the insulin positive cells inside of one of these large islets. This one here shows somatostatin 14, which basically forms a rim around the insulin positive cells. The somatostatin 28 then forms a rim

around the somatostatin 14 positive cells, and then the glucagon positive cells are spindly and scattered throughout. So you can see that the somatostatin 28 cells make up a large component of the islet, but when you transplant these into a mammalian recipient, over a very short period of time, basically, they tend to disappear. And this was data we presented at the Xenotransplant Congress last year, and you can see that the insulin positive cells in the graft are still present, somatostatin 14, the glucagon positive cells, but at 30 days, ah, there are very few of these left, and essentially what we found by about 60 days, there are almost none left. And so it appears as if, basically, the -- the absence of appropriate feedback mechanisms that would be specific for these cells when you transplant them into a foreign environment like a mammal, basically they disappear.

So there is one final major issue, if you were to consider using tilapia as a source for clinical transplantation. Tilapia islets make tilapia insulin, and the comparison here between the A chain and the B chain of tilapia and human insulin molecules show that there is 17 immuno acids that differ between the two peptides. So to address this, we moved into the realm of transgenic fish production. And most of the work that had been done on transgenic fish with large commercially important species were directed at making them grow faster. But we used these techniques to basically make a transgenic tilapia that makes human insulin in its pancreas. And so anyway, here you can see the -- basically the cloned and sequenced tilapia insulin gene with the B chain, the pre-pro-liter, the C peptide containing intron, and the A chain. This was done in my collaborator's lab, Dr. Bill Pohajdak. What Bill then did was by site-directed mutagenesis changed the codons representing the 17 amino acids that differ between tilapia and human insulin. So that we have a tilapia insulin gene that codes from human insulin. Then from there we took the male and female tilapia, we generated fertilized eggs in vitro, then did gene microinjections with this humanized construct.

Here you can see, basically, lanes 11 through 14 are positive and negative controls. We screened simultaneously for the tilapia insulin gene, and then also the humanized tilapia insulin gene. So in this lane six, you can see that we had sufficient DNA to recognize the tilapia insulin gene, and also that we had a positive transgenic.

So progress to date, we've cloned, sequenced and humanized the tilapia insulin gene. We produced small numbers of transgenic tilapia founders with incorporation of the humanized tilapia insulin gene. We've achieved germ line transmission of the humanized insulin transgene in up to 20 percent of the F1 offspring. Using radioimmunoassay for human insulin that does not cross-react with the tilapia insulin, we have shown high levels of circulating humanized insulin in the serum of F1 offspring.

Here you can see a commercial radioimmunoassay. This is F1 offspring from a transgenic male crossed with a wild type female. You can see 21 transgenics and three controls were tested. Of the 21 transgenics, 16 had high insulin levels, one off the standard curve, but 15 within kind of the standard range. And you can see the mean level of insulin in the range at what are control data for humans using the same assay.

So non-fasting blood glucose levels in transgenic tilapia are not significantly different than those in control tilapia, suggesting that insulin secretion is regulated using immunoperoxidase staining, the beta cells in the islets of the transgenic tilapia, but not the control tilapia stained for human insulin.

This next slide shows a section of human pancreas and a section of a control tilapia islet from a juvenile tilapia on the same slide stained by immunoperoxidase for insulin, you can see the presence of insulin in the islets in the human pancreas, and no staining in the tilapia islets.

This is from a transgenic tilapia. You can see once again the positive control, the human pancreas, and you can see clusters of strongly insulin positive cells scattered throughout both this large and this smaller

islet.

The -- As one would expect, using a microinjection technique, we've not created a knockout. And so our fish make both human insulin and tilapia insulin simultaneously. And ideally, what we would like to do is to be able to achieve a knockout, so not just get the gene in, and not just get it functioning, but to actually get it in the right spot. And so we're doing considerable work now on trying to develop an ES cell technology for tilapia, and we have specific funding for that.

So comparison of the transgenic tilapia and the pig islets, the islet characteristics, human-like insulin structure, tilapia, yes. Pigs, yes. Highly glucose responsive, both. High insulin output, both. I didn't show it here, but you can cryopreserve these, freeze them in liquid nitrogen, thaw them and then transplant them, so that is true of both, but where the tilapia are favorable are that they are simple and inexpensive to harvest. They have a low metabolic demand, they don't need much oxygen, and we project that the donor animal production costs would be very low relative to pigs. And the reason for that is that tilapia are 2.5 fold more efficient at converting food into body mass. They produce less waste products per kilogram body mass, shorter generation times. Conception to sexual maturity is six months versus 12 months. They have larger litter sizes, hundreds or thousands, versus less than 10. Shorter minimal intervals between litters, two weeks versus six months, and they have miniscule space requirements for housing. This actually gets to be one of the more important issues.

And, actually, let me backtrack here for a second. So we actually estimate that if you were to -- Based on our best predictions of what you would need in order to do a hundred thousand clinical transplants a year with pigs as donors using an encapsulation device, versus tilapia as donor -- the transgenic tilapia as donors, we estimate that the housing requirements for the pigs would be roughly 200 factories of 20,000 square meters floor space per year to house the pigs; whereas, you'd only need six to eight factories of the same size for the fish. So it actually gets into some extreme cost savings there, in addition to the costs of not having to pay six thousand dollars per islet isolation procedure.

So are there any down sides? I was asked to discuss possible xenozoonoses as well. And tilapia -- actually, fish in general are -- there are no known fish viruses that are infective in man. There are sporadic reports of endogenous retroviruses found in fish. None have been reported in tilapia, but that is probably just because they haven't looked very hard yet.

And most fish diseases that are of a bacterial nature actually relate to fish being raised where there is poor human sanitation, and the fish are in contaminated water. So it's actually reasonably -- or we expect that it would be reasonably easy to clean up bacterial type pathogens, because you can actually take the fish eggs, and there are FDA-approved treatments such as bleach, where since the eggs have kind of a hard shell around them, you can actually treat them with bleach and other kind of antibacterial substances that are approved by FDA already for consumption of food fish. So anyway, there is actually streptococcus iniae was reported in tilapia, and this is one of the specific pathogens that one would have to exclude.

And so in conclusion, we'd also like to try to revolutionize the fast food industry. And one other quick point: Fish don't have alpha-Gal.

DR. SALOMON: Thank you, Jim. That was fantastic. Okay, ah, I think that we're at kind of the point here, the schedule goes that there is some discussion, to 6:55. So that would give us five, ten minutes here just to stay on time, and then I'll turn it back to Megan and our chair, I guess, the three of us will each make sort of a summary comment. But is there any specific discussion or questions on these last two presentations under cell transplantation?

DR. SYKES: I have one. Is the islet structure, and the large islet size of the tilapia unique compared to

other fish? I mean the reason I ask is there are other fish species that people interested in development and so on, use, because of their -- the ease with which they can be genetically modified and genes knocked out, and obviously the tilapia doesn't seem to be that way, from the way you described your efforts at knocking out genes. So would another type of fish be just as good and yet easier to modify?

DR. WRIGHT: Most types of teleost or bony fish, in contrast to sharks that could be cartilagenous fish, will have this same type of pancreas. The species that you're referring to are the zebrafish and the Japanese medaka, both of them are about this big (indicating), so the problem would be using them for transplantation. And actually nobody yet has actually developed a knockout technology in fish. Ah, it's very close. In both zebrafish and in medaka, ES-like cells have been generated. You can take disbursed blastula cells and inject -- microinject them into a developing blastula of a different color fish, like an albino versus a pigmented one, get chimerism, and you can show -- Then if you show pigment chimerism, you can also in a small percentage get germ line expression as well. So all of the different components seem to be there, but nobody has actually yet developed a knockout fish, but it looks like it's probably about that close, and we're hoping to follow along behind them not too far after that.

DR. SALOMON: Could you -- I'm sure you've done this, so could you estimate for us how many fish you would have to actually kill, right, to get a human transplanted?

DR. WRIGHT: Okay, yeah, actually I can do that. If you -- The only data that really exists on where encapsulation of mammalian islets has been done in transplantation into man, ah, would be the stuff done by Patrick Soon Shung back about 10 years ago, and he estimated basically their grafts failed over a period of time. He estimated that you would need 20 thousand islet equivalents per kilogram of recipient in order for it to work. Like I said, his didn't. But if you assume that, then a 70 kilogram patient would require 1.4 million islet equivalents to do a transplant. A 700 gram fish basically gives you 2000 islet equivalents. That is for a non-transgenic fish. The trans -- or -- Tilapia insulin is probably roughly about a third as biologically active as is human insulin, so one would assume that fish making human insulin rather than the tilapia insulin, you would have some savings there of roughly two-thirds, so if you assume that the 2000 islet equivalents per fish, and you need -- So you would need 700 fish, and then, you know, maybe one-third of that basically would be what you would probably actually need. That is also assuming that the -- basically that you have the same pore survival of the fish islets as you do of mammalian islets in these encapsulation devices, since the mammalian islets are highly oxygen sensitive, and the tilapia ones are not, you would expect maybe some additional savings there in that my expectation would be that they would not die off quite as quickly in this kind of hypoxic environment. So it's actually quite feasible, considering that you can -- I myself can harvest about 50 fish an hour, if I were to set my mind so to it. So an untrained technician basically in about five or six hours with little other reagent costs, basically, could potentially do one transplant.

DR. MICHAELS: Could you just reiterate -- I got lost in all the numbers in the harvesting. How many tilapia did you think you would need? And I might -- I just wanted to add that this strep iniae is going to be more of a problem for the harvester than for the recipient of the islet, since it's from the fins.

DR. WRIGHT: Right.

DR. MICHAELS: I'm glad you brought that up.

DR. WRIGHT: The strep iniae -- We've cultured all of our tilapia. We've never seen it. It's only been reported in one episode in Toronto, and there were a couple of episodes where it was found in fish, I think one in Texas, and one maybe Israel, I can't remember for sure. But -- so anyway, we don't expect that that is really a very common pathogen, but because I had that nifty, you know, mad fish disease slide, I thought I would put it in there.

As far as the actual number, we estimate that it would probably be somewhere in the range of maybe around three hundred fish.

DR. SYKES: Question about the oxygen tension that these islets tolerate. While obviously it would be advantageous, using an enlarged encapsulation device or even with free nonvascularized grafts to tolerate low oxygen tension, would higher oxygen tensions, as are present in a normal well-vascularized mammalian environment, actually be deleterious to these grafts? Would they undergo oxygen-induced injury?

DR. WRIGHT: It doesn't seem to be the case. We actually did a study a number of years ago where in the -- Between 1980 and 1990, there was a whole literature on transplanting rat islets into mice in which they were doing all different types of culture techniques to get rid of passenger leukocytes, et cetera. And most of it didn't translate very well into the '90s, when people started looking at large animal donors, and, you know, more discordant transplants. But one of the methods was basically a culture in 95 percent of oxygen, you know, for a week, ah -- a week or two weeks, I can't remember which. But we did that, and we really didn't see any loss in the islet cells. So -- And in fact, we even did it under hyperbaric oxygen conditions for I think three days, because that was another report. And so I don't think that that is probably an issue, but I can't say for sure.

DR. SALOMON: I was going to defer, Harold, to Louisa to --

DR. VANDERPOOL: I think we ought to leave it to our closing comments. We have five minutes, Dan, Megan and then myself.

DR. SALOMON: Actually, I was perfectly serious. I was going to -- but -- So I think that, from my point of view, ah, in terms of this afternoon, what came out for me -- I think that the substance of what we talked about, we've reviewed at each point along the way. The only thing we didn't review was this last thing on cell transplantation. And my feeling was that that is relatively clear in the sense that I don't think it's ready quite yet for many of the reasons that are standing in the way of cell transplantation in general, that the issues are not, and then there is a whole series of issues that are associated with xenotransplantation that Jim, I think, was a perfect model study in his system. And also of clever ways to get around it, like transgenically engineering a human insulin gene, which of course even raises the possibility of raising animals that had two or three times the efficiency of human insulin, by having more beta cells, or cells that, you know, a richer pancreas -- or have more islets per pancreas. All those things are possible, perhaps.

From my point of view, one of the things that I'll carry away is something Robyn and I discussed a little bit, in the last break, and that was now that we have established some sort of a working sense of where the science is, not certainly trying to say that it's all complete in one afternoon, but it's really been a series of beautiful discussions since this morning. Does the committee now, you know, want to do with this? How do we put together an agenda that we're all comfortable with that represents the different interests and perspectives and our sense of the mission of the committee?

And I certainly look forward to, you know, further discussions of that.

DR. SYKES: I won't take too much time, because it's been a long and intensive day; but I think it's been a great day. I think we've heard a lot of exciting science, and we've seen the limitations in every area, the tilapia stuff is fascinating. I have a million questions about the immunology, but there is no time to discuss that right now. But it certainly is something interesting to watch in the future.

I agree with Dan that it's time to evaluate where we go from here as a committee, and, you know, in a discussion with Harold earlier, I think we all would welcome at this point some guidance from the Secretary on what sort of input, what sort of feedback would be desired at this point.

DR. VANDERPOOL: Lily Engstrom and I had a brief conversation about that, and Lily had some very good comments to make, which she can -- she can repeat and expand on tomorrow. I think one of the things I carried away from her and my conversation is we can certainly report on the state of the science on a variety of things and say: This is -- This is what we found. When we move toward recommendations, or requests, at that point, we're -- we're looking at needing to go to and receive perhaps clearance from the Secretary before we, as a committee, would -- would publicize something.

The second comment is we may not be able to be unanimous. We don't have to as a committee. But I think we will be responsible for stating, ah, a -- a non-majority point of view, if that is -- if that also occurs.

My final -- my final comment has to be a touch of relief from a very busy day, because we have learned a great deal, but it is time to end. I want to ask, from one I could gather, the tilapia does have scales, doesn't it, as a fish?

DR. WRIGHT: Oh, yes.

DR. VANDERPOOL: I'm so pleased, because that means that instead of merely talking about pork as the way to sponsor xenotransplantation, we now have something that is actually kosher. End of day.

DR. SYKES: Finally, I'd like to thank everybody, the speakers, once again, and all the committee members for all their valuable input. Thank you.